Heart Sarcolemmal ($Na^+ + K^+$)-ATPase Has an Essential Amino Group in the Potassium Binding Site on the Enzyme Molecule

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Abstract. Selective modification of primary amino groups of ($Na^+ + K^+$)-ATPase by trinitrobenzene sulfonic acid (TNBS) resulted in a considerable inhibition of the specific activity of the enzyme. Investigation by means of enzyme and sorption kinetics of activation of heart sarcolemmal ($Na^+ + K^+$)-ATPase by its monovalent cationic ligands added simultaneously with TNBS revealed: i) a considerable competition between K^+-ions and TNBS for the potassium binding site on the enzyme molecule; ii) a non-competitive type of inhibition of Na^+-induced activation of the enzyme. Both, potassium and sodium ions depressed, and magnesium ions enhanced the initial rate of TNBS-sorption; however, none of the above cations influenced the equilibrium value of TNBS sorption onto isolated sarcolemmal membranes. Ouabain, on the other hand, did not inhibit the initial rate and decreased the equilibrium value of TNBS sorption onto the membranes. The results obtained enabled the identification of an essential amino group in the potassium binding site of the ($Na^+ + K^+$)-ATPase molecule.

Key words: Heart sarcolemma — K^+-binding site of ($Na^+ + K^+$)-ATPase — Amino group — Trinitrobenzene sulfonic acid

Introduction

It has been well documented that the active transport of sodium and potassium ions across the plasma membrane proceeds under the action of ($Na^+ + K^+$)-ATPase (ATP-phosphohydrolase E.C. 3.6.1.3). This enzyme has been shown to contain in its active site an essential sulfhydryl group (Patzelt-Wenczler and Schoner 1981; Ziegelhöffer et al. 1983; Džurba et al. 1985) in addition to essential aspartic (Jørgensen 1982) and tyrosine (Cantley at al. 1978) groups. However, relatively little is known about the structure and/or essential functional groups of ($Na^+ + K^+$)-ATPase molecule in its binding sites for sodium and potassium. Monovalent

cations may react with proteins by forming both, ionic and coordination bounds. A typical place for the latter kind of interaction may represent the amino group of lysine (Hughes 1981). Indeed, modification of NH_2 group on purified $(Na^+ + K^+)$-ATPase from the rabbit kidney outer medulla by 2,4,6-trinitrobenzene sulfonic acid (TNBS) yielded a significant inhibition of the enzyme activity (DePont et al. 1984).

The aim of the present paper was: i) to demonstrate the inhibitory effect of TNBS on $(Na^+ + K^+)$-ATPase in a partially purified sarcolemmal fraction from rat heart and to extend the knowledge about the mechanism of this inhibition; ii) to contribute to knowledge about the particular role of amino groups in the reaction mechanism of $(Na^+ + K^+)$-ATPase.

Materials and Methods

Membrane preparation

Rat heart membrane preparation enriched in sarcolemma was isolated by a procedure combining the method of hypotonic shock with subsequent NaI treatment (Vrbjar et al. 1984). The resulting preparation contained less than 3 per cent of other subcellular membrane particles (Kostka et al. 1981). Adult male Wistar albino rats were used in all experiments.

Estimation of $(Na^+ + K^+)$-ATPase activity

Specific activity of sarcolemmal $(Na^+ + K^+)$-ATPase was established as the difference between the amount of orthophosphate liberated from ATP splitting in the presence or absence of both sodium and potassium ions (100 mmol . l^{-1} and 10 mmol . l^{-1} respectively). The enzyme was let to react for 15 min at 37 °C in 1 ml of reaction medium containing imidazol-HCl buffer (30 mmol . l^{-1}) pH = 7.0, $MgCl_2$ (2 mmol . l^{-1}) and 30—60 μg membrane proteins. The reaction was started by adding ATP (final concentration 2 mmol . l^{-1}) and it was terminated by adding 1 ml of trichloroacetic acid (0.73 mol . l^{-1}). TNBS, when used, was added together with the cationic ligands 10 min before starting the enzyme reaction. Specific activity of the enzyme was expressed in μmoles P_i . h^{-1} . mg^{-1} of membrane proteins. The free orthophosphate and protein concentrations were determined according to Taussky and Shorr (1953) and Lowry et al. (1951), respectively. Various amounts of TNBS *(50—100* μmol . l^{-1}) were allowed to interact with isolated sarcolemmal membranes for 10 min under identical conditions as used for enzyme reaction prior to its triggering by the addition of ATP. The estimation of sarcolemmal Ca^{2+}-ATPase, (Mg—Ca)-ATPase and Mg^{2+}-ATPase activities as well as oligomycine-sensitivity of the latter were described in detail in our previous paper (Ziegelhöffer et al. 1983).

Kinetics of $(Na^+ + K^+)$-ATPase stimulation by monovalent cations

Stimulation of $(Na^+ + K^+)$-ATPase activity with increasing concentrations of Na^+ or K^+ ions was investigated within the range of 3—100 mmol . l^{-1} and 0.33—10 mmol . l^{-1} respectively. Reaction conditions and concentrations of all other reactants were kept similar to those used in estimation of the enzyme activity. The results obtained were plotted in a double reciprocal system. Kinetic parameters were evaluated from the linear parts of the plots, i.e. for ligand concentrations exceeding 0.5 and 5.0 mmol . l^{-1} for potassium and sodium activation, respectively, where the cooperativity between the respective cation binding sites was not yet manifested.

Kinetics of TNBS sorption onto membranes

Binding of TNBS onto the membranes was investigated by incubating a constant amount of sarcolemmal fraction (80 μg of membrane proteins) in 0.5 ml reaction medium consisting of imidazol-HCl (30 mmol . l^{-1}) buffer pH = 7.0, containing 200 μmol . l^{-1} TNBS in the presence or absence of each: NaCl (100 mmol . l^{-1}), KCl (10 mmol . l^{-1}), $MgCl_2$ (2 mmol . l^{-1}) or 2 mmol . l^{-1} of ouabain. After various time intervals (5—60 min) the binding was stopped by the addition of 1 ml of 0.1 mol . l^{-1} HCl. The amounts of bound TNBS were assessed by the direct spectrophotometric method according to DePont et al. (1984) at 340 nm using a double beam spectral photometer Beckman DB-G. The course of TNBS sorption kinetics onto the sarcolemmal membrane was established by means of equation (1) derived in our previous paper (Breier et al. 1984).

$$B = B_e \cdot t \cdot (t + t_{1/2})^{-1} \quad (1)$$

where B represents the amount of adsorbed substance over time t,
B_e is the equilibrium sorption over time $t \to \infty$,
$t_{1/2}$ is the sorption half-time.
Transformation of the obtained experimental data into double reciprocal plot yielded a straight line with an intercept on the ordinate equal to B_e^{-1}, and a slope equal to V_0^{-1}. The symbol V_0 representing the initial rate of sorption was found to be defined by the ratio $B_e/t_{1/2}$.

Materials

TNBS was obtained from FLUKA; ATP, ouabain and imidazol from Boehringer (all FRG). Other reagents were purchased from Lachema (Czechoslovakia). All chemicals were of analytical grade.

Results

The investigated sarcolemmal preparation was characterized by the following specific activities of enzymes: $(Na^+ + K^+)$-ATPase 9.99 ± 0.67, Mg^{2+}-ATPase 21.41 ± 0.93 and Ca^{2+}-ATPase with low affinity to calcium 19.26 ± 0.86 μmoles P_i . h^{-1} . mg^{-1} of membrane proteins. The preparation showed an only very low (Mg—Ca)-ATPase activity (0.23 ± 0.19 μmoles P_i . h^{-1} . mg^{-1} of membrane proteins), thus disproving any considerable contamination by sarcoplasmic reticulum membranes or myofibrils. A sensitivity of Mg^{2+}-ATPase to oligomycine of less than 2 % characterized the possible contamination by mitochondrial membranes.

Trinitrobenzene sulfonic acid in concentrations between 50—1000 μmol . l^{-1}, applied simultaneously with the cationic ligands, considerably inhibited the specific activity of $(Na^+ + K^+)$-ATPase with an ID_{50} value of 395 μmol TNBS . l^{-1}. The classical Dixon plot of this inhibition is shown in Fig. 1. A further kinetic study of stimulation of $(Na^+ + K^+)$-ATPase activity by its monovalent cationic ligands in the presence or absence of TNBS (0.1 mmol . l^{-1}) revealed: i) a combined type of inhibition of enzyme activation by potassium ions characterized by a 33 per cent decrease of the V^K_{max} value, and by an increase of the $K^K_{0.5}$ value from 0.260 to 0.975 mmol . l^{-1} (Fig. 2); ii) a non-competitive type of inhibition of $(Na^+ + K^+)$-ATPase activation by sodium ions with a considerable decrease of the V^{Na}_{max} value by 40 % (Fig. 3).

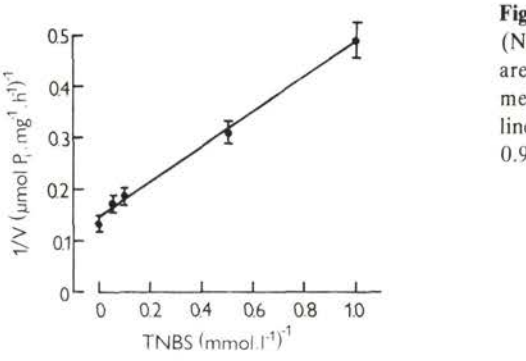

Fig. 1. Dixon plot of inhibition of sarcolemmal ($Na^+ + K^+$)-ATPase activity by TNBS. Results are expressed as means ± S.E.M. from 9 separate measurements. The straight line was drawn by linear regression ($V^{-1} = 0.352 \cdot c_{TNBS} + 0.138$; $r = 0.993$).

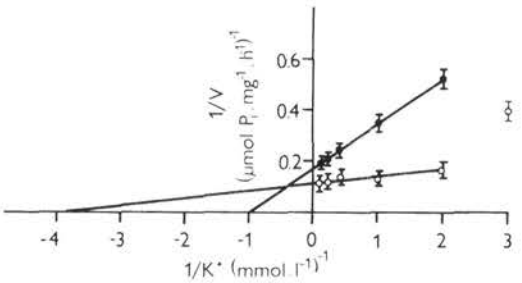

Fig. 2. Inhibitory effect of TNBS on stimulation of sarcolemmal ($Na^+ + K^+$)-ATPase activity by increasing amounts of potassium. The straight lines were drawn by linear regression using only data from the linear parts of the respective Lineweaver-Burk plots i.e., for potassium concentrations $[K^+] \geqq 0.5$ mmol . l^{-1}. Results are means ± S.E.M from 9 separate measurements; ○-without TNBS, ●-with TNBS (100 μmol . l^{-1}). The regression line for the non-inhibited enzyme is characterized by equation $V^{-1} = 0.112 + 0.029 \cdot [K^+]^{-1}$ and parameters $r = 0.992$, $V^K_{max} = 8.93$ μmol P_i . mg^{-1} . h^{-1} and $K^K_{0.5} = 0.259$ mmol . l^{-1}. For the regression line for the inhibited enzyme the equation $V^{-1} = 0.178 + 0.173 \cdot [K^+)^{-1}$ and parameters $r = 0.994$, $V^K_{max} = 5.617$ μmol P_i . mg^{-1} . h^{-1} and $K^K_{0.5} = 0.952$ mmol . l^{-1} were found to be characteristic.

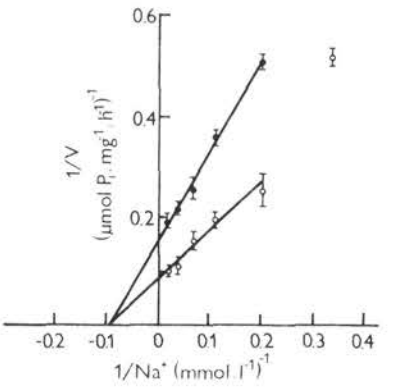

Fig. 3. Inhibitory effect of TNBS on stimulation of sarcolemmal ($Na^+ + K^+$)-ATPase activity by increasing amounts of sodium. The straight lines were drawn by linear regression using only data from the linear parts of the respective Lineweaver-Burk plots i.e. for sodium concentrations $[Na^+] \geqq 5$ mmol . l^{-1}. Results are means ± S.E.M. from 9 separate measurements; ○-without TNBS, ●-with TNBS (100 μmol . l^{-1}). The regression line for the non-inhibited enzyme is characterized by equation $V^{-1} = 0.089 + 0.967 \cdot [Na^+]^{-1}$ and parameters $r = 0.998$, $V^{Na}_{max} = 11.235$ μmol P_i . mg^{-1} . h^{-1} and $K^{Na}_{0.5} = 10.865$ mmol . l^{-1}. For the regression line for the inhibited enzyme the equation $V^{-1} = 0.160 + 1.74 \cdot [Na^+]^{-1}$ and parameters $r = 0.991$, $V^{Na}_{max} = 6.250$ μmol P_i . mg^{-1} . h^{-1} and $K^{Na}_{0.5} = 10.875$ mmol . l^{-1} were found to be characteristic.

The kinetics of TNBS sorption onto isolated sarcolemmal membranes under control conditions, i.e. in the absence of cations and ouabain, was characterized by the following values: equilibrium sorption $B_e = 0.899 \pm 0.070$ μmoles of bound

TNBS per mg of membrane proteins; initial rate of sorption $V_0 = 7.069 \pm 0.682$ μmoles of bound TNBS per mg of membrane proteins per hour (Table 1). In the presence of K^+ (10 mmol . l^{-1}) or Na^+ (100 mmol . l^{-1}) ions a considerable decrease of the V_0 value of TNBS sorption could be observed. Contrary to the above monovalent cations, magnesium (2 mmol . l^{-1}) was found to increase the V_0 value of TNBS binding. However, none of these cations affected the B_e value of TNBS binding (Fig. 4, Table 1). On the other hand, ouabain (2 mmol . l^{-1}) markedly decreased the B_e value, leaving the initial rate of TNBS sorption essentially unchanged (Fig. 5, Table 1).

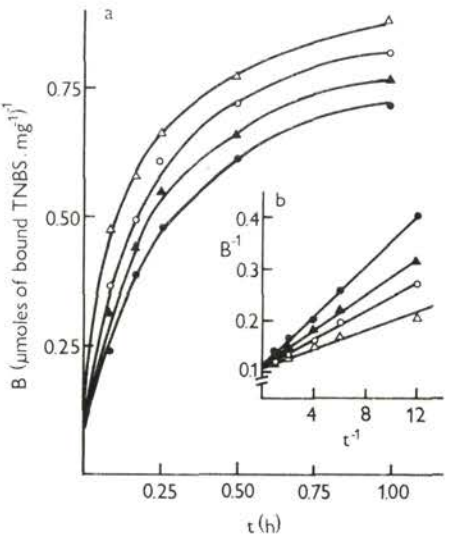

Fig. 4. Effects of sodium, potassium and magnesium ions on the kinetics of TNBS sorption onto the isolated sarcolemmal membranes. *a*: Direct plotting of the sorption kinetics. *b*: Double reciprocal plot of the sorption kinetics. Symbols: O-TNBS sorption in absence of cations, ● — in the presence of KCl (10 mmol . l^{-1}), △ — in the presence of $MgCl_2$ (2 mmol . l^{-1}) and ▲ — int the presence of NaCl (100 mmol . l^{-1}). Results in the abscissa are given as $B^{-1} . 10^{-1}$. The respective parameters of sorption kinetics with a confidence interval of 95 % determined from 6 individual values are shown in Table 1.

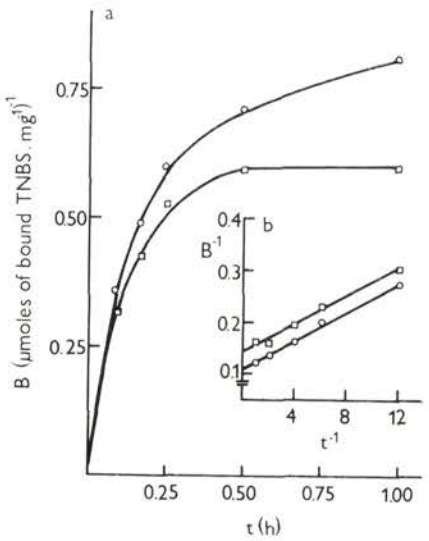

Fig. 5. Effect of ouabain on the kinetics of TNBS sorption onto the isolated sarcolemmal membranes. *a*: Direct plotting of the sorption kinetics. *b*: Double reciprocal plot of the sorption kinetics. Symbols: O — TNBS sorption in the absence and □ — in the presence of ouabain (2 mmol . l^{-1}). Results in the abscissa are given as $B^{-1} . 10^{-1}$. The obtained parameters of sorption kinetics with a confidence interval of 95 % determined from 6 individual values are shown in Table 1.

Table 1. Effects of sodium, potassium and magnesium ions and ouabain on the kinetic parameters of TNBS sorption.

Additions	B_e (μmoles of bound TNBS . mg^{-1}) . 10	V_0 (μmoles of bound TNBS . h^{-1} . mg^{-1}) . 10	$t_{0.5}$ (*h*)	*r*
none	8.99 ± 0.70	70.69 ± 6.82	0.130 ± 0.011	0.992
KCl (10 mmol . l^{-1})	9.02 ± 0.68	40.75 ± 4.74	0.170 ± 0.015	0.998
NaCl (100 mmol . l^{-1})	9.05 ± 0.85	57.74 ± 5.13	0.149 ± 0.012	0.995
$MgCl_2$ (2 mmol . l^{-1})	8.92 ± 0.82	112.59 ± 9.25	0.082 ± 0.007	0.989
ouabain (2 mmol . l^{-1})	6.88 ± 0.85	73.37 ± 6.90	0.092 ± 0.008	0.993

Results are expressed as the computed values ± 95 % confidence interval. The methods used for calculations have been described in our previous paper (Breier et al. 1984). *r* — correlation coefficient of the regression analysis of double reciprocal linearization.

Discussion

It was reported by Wand et al. (1978) that at neutral pH values TNBS selectively reacts with amino groups by forming trinitroanilides, and that this reaction may be used for the quantitative determination of primary NH_2 group content, even in insoluble materials. In experiments with ATP present, TNBS may also react with the vicinal diol group on the ribose moiety of ATP. Nevertheless, this reaction is very slow, and it was found to occur only at values of pH>9.0 (Hiratsuka and Uchida 1973). Although the product of the latter reaction was proved to be a competitive inhibitor of $(Na^+ + K^+)$-ATPase(Moczydłowski and Fortes 1981), its formation under our experimental conditions was so negligible that its presence could not be detected by UV and visual spectroscopy at 259, 408 and 470 nm.

Our results confirmed that, similarly to $(Na^+ + K^+)$-ATPase from the dog kidney outer medulla (DePont et al. 1984), TNBS inhibited the specific activity of the above enzyme also in the isolated cardiac sarcolemma. A kinetic study of activation of $(Na^+ + K^+)$-ATPase by increasing concentrations of K^+ ions in the presence of TNBS revealed a combined type of inhibition of the above process, characterized by considerably decreased V^K_{max} and increased $K^K_{0.5}$ values. This suggested a significant lowering of affinity to potassium of the respective binding sites on the enzyme molecule. The observed decrease in maximal reaction rate for potassium activation might easily be caused by interaction of TNBS in parts of the $(Na^+ + K^+)$-ATPase molecule distant from the potassium binding site, since it has been shown that the enzyme contains 98 amino groups per molecule (Peters et al.

1981). The TNBS-induced non-competitive type of inhibition of $(Na^+ + K^+)$-ATPase activation by sodium ions may be attributed to the absence of an essential amino group in the sodium binding site and/or by inaccessibility of this particular locus on the enzyme for TNBS. As it appeared from the results shown in Fig. 4, both potassium and sodium competitively inhibited whereas magnesium facilitated the sorption of TNBS onto the membranes. This will manifest itself in a protection of the $(Na^+ + K^+)$-ATPase activity against inhibition by TNBS in the presence of potassium, and on the other hand, in a potentation of enzyme inhibition in the presence of magnesium as it was recently shown by DePont et al. (1984). The discussed antagonistic action of potassium and magnesium in respect to $(Na^+ + K^+)$-ATPase inhibition by TNBS points to an interaction of the inhibitor with a particular amino group in the potassium binding site on the enzyme molecule. This suggestion seems to be plausible the more that the binding of magnesium onto the sarcolemma (potentiating the enzyme inhibition by TNBS) alone was found to induce such a change in the secondary structure of membrane proteins which corresponds to preferential establishment of the E_2 conformation form of the $(Na^+ + K^+)$-ATPase molecule, characterized by high affinity to potassium (Vrbjar et al. 1984).

Although sodium ions inhibited TNBS sorption onto the sarcolemmal membranes, they had, however, no protective effect against the TNBS-induced inhibition of the $(Na^+ + K^+)$-ATPase activity. Hence, the observed influence of sodium ions on TNBS binding might not be considered as specific in respect to the above enzyme.

An interaction of TNBS directly in the potassium binding site of the $(Na^+ + K^+)$-ATPase molecule has also been suggested by other results obtained with ouabain. The latter was namely found to decrease considerably the B_e value for TNBS sorption without any effect on the initial rate of sorption. This speaks against any competition between TNBS and ouabain for the same binding site, rather suggesting a decrease in the number of binding sites able to react with TNBS. Hence, ouabain obviously modulated the sorption of TNBS in an allosteric manner, absolutely similar to that when modulating potassium binding to the molecule of $(Na^+ + K^+)$-ATPase (Lüllman et al. 1975; Hansen 1984).

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