Short communication

Modification of Primary Amino Groups in Rat Heart Sarcolemma by 2,4,6-Trinitrobenzene Sulfonic Acid in Respect to the Activities of $(Na^+ + K^+)$-ATPase, Na^+-ATPase and *p*NPPase. Function of the Potassium Binding Sites

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$(Na^+ + K^+)$-ATPase has been shown to loose its specific activity following modification of primary amino groups on the enzyme molecule with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (DePont et al. 1984; Breier et al. 1986). The inhibition induced by TNBS was strengthened or weakened in the presence of magnesium or potassium ions. Particularly responsible for the above inhibition was made an interaction of TNBS with the potassium binding site on the $(Na^+ + K^+)$-ATPase molecule (Breier et al. 1986), thus confirming the presence of an essential primary amino group in the above locus. Such a conclusion seems to be plausible the more that TNBS was inhibitory also to K^+-stimulated *p*-nitrophenylphosphatase (K^+-pNPPase) activity (DePont et al. 1984) which is predominantly manifested at conformation E_2 of $(Na^+ + K^+)$-ATPase (Robinson 1984). However, Na^+-stimulated, Mg^{2+}-dependent hydrolysis of ATP (Na^+--ATPase activity) which was attributed to prevailing conformation E_1 of $(Na^+ + K^+)$-ATPase (Vrbjar et al. 1984) has not yet been studied in respect to TNBS inhibition.

The aim of the present paper was:
i) to study the effect and mode of interaction of TNBS with Na^+-ATPase in rat heart sarcolemma (SL); ii) to study interferences of potassium and magnesium ions with TNBS-induced modulation of heart SL $(Na^+ + K^+)$-ATPase, Na^+--ATPase and K^+-*p*NPPase activities.

Rat heart SL was isolated using a combination of hypotonic shock with NaI treatment as described elsewhere (Vrbjar et al. 1984). The SL fraction was

contaminated to less than 3 per cent by other subcellular membrane particles such as sarcoplasmic reticulum, mitochondria and myofibrils (Breier et al. 1986).

Determination of enzyme activities. Specific activity of SL ($Na^+ + K^+$)-ATPase was established as the difference between the amounts 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 activity of Na^+-ATPase was established in an analogical way, however, in the absence of potassium.

Enzyme reaction lasting 15 min was run at 37 °C in 1 ml of reaction medium containing 30 mmol.l^{-1} imidazol-HCl buffer (pH = 7.0), 2 mmol.l^{-1} $MgCl_2$ and 30—60 μg of membrane protein. The reaction was started by addition of ATP (final concentration 2 mmol.l^{-1}) and it was terminated by 1 ml of trichloracetic acid (0.73 mol.l^{-1}). The orthophosphate concentration was assesed by the method of Taussky and Shorr (1953).

K^+-*p*NPPase activity was established as the difference between the amounts of *p*-nitrophenyl (*p*NP) liberated from *p*-nitrophenylphosphate (*p*NPP) in the presence or absence of potassium (5 mmol.l^{-1}). The enzyme reaction was started by addition of *p*NPP (final concentration 2 mmol.l^{-1}). The same reaction medium as described for the estimation of ($Na^+ + K^+$)-ATPase activity was used. After a time interval of 15 min the reaction was stopped by the addition of 1 ml NaOH (0.1 mol.l^{-1}). The amounts of *p*NP liberated were monitored spectrophotometrically, directly in the reaction medium at 410 nm. TNBS was left to interact with membrane proteins at pH = 7.0 in a medium containing 10 mmol TRIS-HCl, 400—600 μg of membrane proteins and 50—500 nmol of TNBS per 1 ml in the presence or absence of Mg^{2+} (2 mmol.l^{-1}), K^+ (10 mmol.l^{-1}) as well as *p*NPP (5 mmol.l^{-1}). After a reaction time of 30 min at room temperature and intermittent stirring, the suspension was spun down for 10 min at 3000 × *g*. The pellet was then resuspended in 10 mmol.l^{-1} TRIS-HCl buffer (pH = 7.0) and adjusted to a protein concentration of 400—600 μg per ml.

The protein concentration was determined according to Lowry et al. (1951), with bovine serum albumin as a standard. Spectrophotometric measurements were performed on a Beckman DBG double beam spectral photometer.

Kinetics of Na^+-ATPase stimulation by sodium. Stimulation of Na^+-ATPase activity with increasing concentrations of sodium was investigated over a range of 25—100 mmol NaCl.l^{-1}. All other reactants used and/or experimental conditions were similar to those required for an optimal assay of the enzyme activity. TNBS was allowed to interact with membrane proteins in the reaction medium at 37 °C for 10 min prior to starting the enzyme reaction by the addition of ATP.

Table 1. Effect of pretreatment with TNBS on the specific activities of $(Na^+ + K^+)$-ATPase and Na^+-ATPase

No	Dose of TNBS μmol TNBS . mg^{-1}	Pretreatment	Na^+-ATPase	$(Na^+ + K^+)$-ATPase
			μmol P_i . h^{-1} . mg^{-1}	
I	—	—	5.26 ± 0.56[a]	12.82 ± 0.74[a]
II	1.124	—	1.77 ± 0.47*	2.47 ± 0.31*
III	0.111	—	5.55 ± 0.41	5.95 ± 0.42*
IV	0.111	$MgCl_2$ (2 mmol . l^{-1})	5.56 ± 0.65	6.07 ± 0.40*
V	0.111	$MgCl_2$ (2 mmol . l^{-1}) KCl (10 mmol . l^{-1})	5.45 ± 0.48[a]	7.82 ± 0.46[a b]*

* Significantly different from the control (No I) at $p < 0.005$

[a] Differences between Na^+-ATPase and $(Na^+ + K^+)$-ATPase activities significant at $p < 0.01$

[b] Significantly different from the values in groups No III and IV ($p < 0.01$)

Experimental data were evaluated by means of the Student's *t*-test.

Materials. TNBS was obtained from Fluka (GFR), ATP, pNPP and imidazol from Sigma (USA). The remaining reactants were purchased from Lachema (Czechoslovakia). All chemicals were of analytical grade.

Rat heart SL preparation was characterized by following specific activities of $(Na^+ + K^+)$-ATPase Na^+-ATPase and K^+-*p*NPPase 12.86 ± 0.84 and $5.26 \pm 0.56\,\mu mol \cdot P_i \cdot mg^{-1} \cdot h^{-1}$, and $4.81 \pm 0.13\,\mu mol\,pNP \cdot mg^{-1} \cdot h^{-1}$, respectively. Other properties of the membrane preparation used have been described in previous papers (Ziegelhöffer et al. 1983; Vrbjar et al. 1984; Džurba et al. 1985; Breier et al. 1986).

A pretreatment with TNBS ($1.124\,\mu mol$ per mg of membrane protein) induced a significant depression ($p < 0.005$) of both $(Na^+ + K^+)$-ATPase and Na^+-ATPase activities and a considerable decrease in the capability of Na^+-ATPase of being further stimulated by potassium (Table 1). An investigation of the above effect in respect to the Na^+-ATPase activity revealed an uncompetitive type of inhibition, thus suggesting that the interaction of the inhibitor will occur in a locus distant from the sodium binding site on the enzyme molecule (Fig. 1). A typical value of K_i of $11.926\,\mu mol\,TNBS \cdot mg^{-1}$ of membrane protein was found for this inhibition.

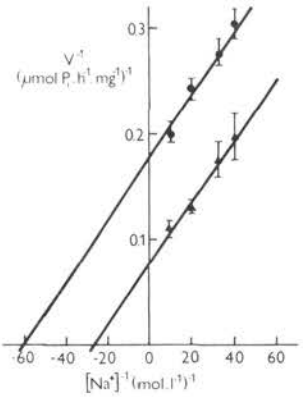

Fig. 1. Lineweaver-Burk plot of stimulation of Na^+-ATPase by increasing concentrations of Na^+ in the presence (●) or absence (▲) of TNBS ($1.273\,\mu mol \cdot mg^{-1}$ of membrane protein). Single points are means ± S. E. M. from 6 different measurements.

On the other hand, on preincubation with a TNBS concentration approximately ten times lower ($0.111\,\mu mol \cdot mg^{-1}$ of membrane protein) and also below it (not shown) the effect of the substance turned to be selective, inhibiting exclusively and completely the K^+-stimulability of the $(Na^+ + K^+)$-ATPase activity, whereby the capacity of the enzyme to be stimulated by sodium i.c. the Na^+-ATPase activity remained fully preserved (Table 1).

The selective inhibitory effect of TNBS could not be modulated by the presence of $2\,mmol \cdot l^{-1}\,MgCl_2$ during the pretreatment; however, when $10\,mmol \cdot l^{-1}$ KCl were added to the preincubation medium in addition to $MgCl_2$

the capacity of $(Na^+ + K^+)$-ATPase to be stimulated by potassium ions remained partially preserved (see Table 1, group V $p < 0.01$).

Magnesium ions were found to enhance both, the initial velocity of TNBS sorption onto SL membrane (Breier et al. 1985), and the inhibition by TNBS of the $(Na^+ + K^+)$-ATPase activity (DePont et al. 1984). This indicates that the inhibitor does not interact in the ATP-binding site of the enzyme, since otherwise magnesium would prevent the inhibition as it could be demonstrated earlier using the Woodward's reagent K (Mardh 1982) or *p*-bromophenylisothiocyanate (Ziegelhöffer et al. 1983) as inhibitors. An essential TNBS-accessible primary amino group was, however, recently found to be present in the potassium binding site of the $(Na^+ + K^+)$-ATPase by ourselves (Breier et al. 1986). The finding that the inhibition by TNBS (10^{-7} mol . mg^{-1} protein) of K^+-stimulation of the above enzyme can be abolished to a considerable degree by the presence of potassium ions, again confirms that the inhibitor in the dose used and below it reacts selectively with an essential NH_2-group in the potassium binding site of $(Na^+ + K^+)$-ATPase. This explanation is also supported by the considerations and results reported by Nagata and Aida (1984). These authors investigated the properties of specific sites for binding of monovalent cations in ion transport systems such as ionic channels, particularly from the aspect of the energetics of binding. They concluded that the coordinatory interaction of potassium ion with the ammonium is most advantegeous in comparison to that with other neutral solvent molecules. Moreover, the selectivity of TNBS reaction in the potassium binding sites of $(Na^+ + K^+)$-ATPase occurs in the

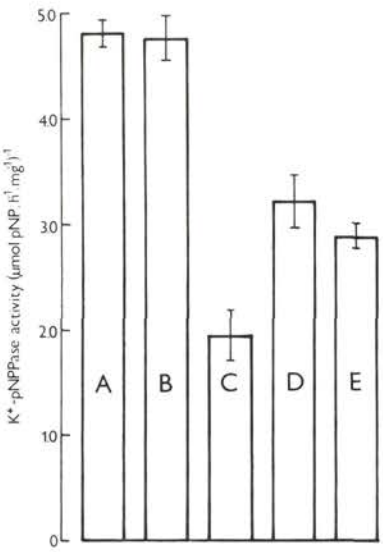

Fig. 2. Effect of TNBS on heart sarcolemmal K^+-*p*NPPase activity. Results are expressed as means ± S.E.M. form 9 different measurements. *A* — control; *B*, *C*, *D* and *E* — membranes pretreated for 30 min with: TNBS (1.610 μmol . mg^{-1} of membrane protein); — TNBS and Mg^{2+} ions (2 mmol . l^{-1}); — TNBS, Mg^{2+} and K^+ ions (10 mmol . l^{-1}) and with TNBS, Mg^{2+} ions and *p*NPP (5 mmol . l^{-1}) respectively. For other conditions, see the text.

presence of NH_2-group with an affinity to TNBS exceeding 30 times* that of other NH_2-groups which participate in the manifestations of Na^+-ATPase activity.

In contrast to the inhibition of K^+-activation of $(Na^+ + K^+)$-ATPase, the inhibition of K^+-*p*NPPase activity by TNBS proved to be of low effectivity and required the presence of magnesium ions; moreover it could be prevented by K^+-ions or *p*NPP (Fig. 2). This seems to indicate that the inhibitor may act similarly in the K^+-binding sites of the enzyme; nevertheless, these K^+-binding sites will have different properties than of $(Na^+ + K^+)$-ATPase. The latter finding may be considered as a further difference between $(Na^+ + K^+)$-ATPase and K^+-*p*NPPase, in addition to those described ty Fleary et al. (1985).

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Final version accepted February 21, 1986

* $K_i = 0.319\,\mu mol\,TNBS \cdot mg^{-1}$ protein, computed from Breier et al. (1986) for the inhibition by TNBS of K^+-stimulation of $(Na^+ + K^+)$-ATPase.