Competitive Inhibition of (Na/K)–ATPase by Furylethylenes with Respect to Potassium Ions

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Abstract. The effects of newly synthetized derivatives of furylethylene: i) 1-(5-nitro-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene (FE1), ii) 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene (FE2), iii) 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-tienocarbonyl ethylene (FE3), on the reaction kinetics of the dog kidney (Na/K)-ATPase were tested. Besides the conjugated triene moiety of the furylethylene skeleton, the groups responsible for the reaction with nucleophilic groups, the formyl group that connects the second furyl ring in FE1 and FE2 and the formyl group that connects the thienyl ring to the furylethylene moiety in FE3. Among the furylethylenes tested, only FE1 was found to react effectively with β-mercaptoethanol (βME) and glycine (GLY) as model substances containing nucleophilic groups, and also exhibit an inhibitory interaction with the (Na/K)-ATPase. A suppression of the reactivity of the formyl group due to the replacement of the furyl ring with the more aromatic thienyl ring in FE3 did not induce any significant change in the reactivity of the compound with the model substances or with (Na/K)-ATPase.

On the other hand, replacement of the NO_2 group on the furylethylene moiety (in FE1) by the less electron-attracting phenylsulfonyl group (in FE2 and FE3) yielded a considerable suppression of the inhibitory effect on (Na/K)-ATPase. Moreover, in comparison to FE1, FE2 and FE3 were found to react less potently with the model nucleophilic substances. The results indicated that the conjugated triene moiety on the furylethylene part of the molecule of FE1 may be made responsible for the inhibitory interaction with the nucleophilic aminoacid residue on the (Na/K)-ATPase-molecule.

FE1 interfered competitively with the (Na/K)-ATPase activation by increas-

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ing amounts of potassium. This was manifested by a significant increase in the apparent $K_{0.5}^{App}$ value and a decrease in the apparent cooperativity constant, n^{App}, for potassium ions, but had no influence on the apparent V_{max}^{App} value for potassium. With respect to the activation of the enzyme with sodium ions and ATP, only FE1 decreased the V_{max}^{App} values while having no considerable influence on the other kinetic variables. It was concluded that FE1 inhibits the (Na/K)–ATPase by selective interaction with some essential nucleophilic (probably SH and/or NH_2) aminoacid residues located in, or closed to the potassium binding site of the enzyme molecule.

Key words: (Na/K)-ATPase — Furylethylenes — Electrophilic reagents — Enzyme kinetics — Cation binding site

Introduction

Because of its strong involvement in the transport of sodium and potassium across the plasma membrane in different types of cells, the sodium- and potassium- stimulated, and magnesium-dependent adenosinetriphosphate hydrolase (E.C. 3.6.1.3): (Na/K)–ATPase (Skou and Esmann 1992) has been one of the most frequently studied enzyme systems. Several electrophilic reagents such as fluorescein isothiocyanate (Carilli et al. 1982), *p*-bromophenyl isothiocyanate (Ziegelhöffer et al. 1983) or 2,4,6-trinitrobenzenesulfonic acid (De Pont et al. 1984) have been shown to inhibit this enzyme by attacking its molecule at diverse sites.

Inhibition studies with isothiocyanates have contributed to the clarification of the chemical composition of the ATP-binding site of the Na/K-ATPase (Carilli et al. 1982; Ziegelhöffer et al. 1983). The inhibitor was first believed to interact selectively with an NH_2 group located at the active site of (Na/K)–ATPase (Farley et al. 1984; Kirley et al. 1984; Ohta et al. 1985). However, this opinion was later questioned, based on data about the reactivity of isothiocyanates with the SH and NH_2 groups (Drobnica and Gemeiner 1976; Drobnica et al. 1977; Gemeiner and Drobnica 1979). For, in the meantime, besides the NH_2 group also an essential SH group present at the ATP binding site of the enzyme, may be considered as a suitable place for FITC attack to the (Na/K)–ATPase (Breier et al. 1989, 1996). This particularly concerns the reaction conditions at pH~7 (Ziegelhöffer et al. 1983; Breier et al. 1995).

It has been suggested previously (Breier et al. 1986, 1987, 1988; Ziegelhöffer et al. 1986) that the binding sites for monovalent cations on the (Na/K)-ATPase molecule contain nucleophilic amino acid residues which, when attacked by electrophilic reagents, may cause inhibition of the enzyme activity. The effect of the latter type of interactions has been verified by means of 2,4,6-trinitrobenzenesulfonic acid, a selective amino reactant (Wand et al. 1978), which proved to interfere with

the stimulation of (Na/K)–ATPase by potassium ions (Breier et al. 1986, 1987). Other electrophilic reagents, such as 5-nitro-2-furylethylene derivatives, which provide numerous addition or substitution reactions with low molecular weight thiols, amines and alcohols (Rosenberg et al. 1985; Baláž et al. 1987), may also attack the (Na/K)-ATPase. A typical representative of this group is 1-(5-nitro-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene (FE1), which contains two electrophilic structures in its molecule: i.) the conjugated triene moiety of the furylethylene skeleton, ii.) the formyl group representing the connection to the second furyl ring has recently been synthesized and described (Špirková et al. 1991). When incubated with (Na/K)-ATPase, FE1 was found to act in an ouabain-like manner, by interfering with the stimulation of (Na/K)-ATPase by potassium (Vrbanová et al. 1994; Breier et al. 1995).

The following goals were followed in the present study: a) verification of the inhibitory effect of FE1 on (Na/K)-ATPase, observed in partially purified enzyme preparations from guinea pig heart sarcolemma (Vrbanová et al. 1994; Breier et al. 1995) as well as in highly purified enzyme preparations from dog kidney, b) identification of the part of the FE1 molecule which may be responsible for the inhibition of the (Na/K)-ATPase. For the latter goal, the effect of FE1 was compared with those of two other purposefully selected furylethylene derivatives: 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene (FE2) and 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-tienocarbonyl ethylene (FE3).

Abbreviations: FE1 – 1-(5-nitro-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene; FE2 – 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene; FE3 – 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-tienocarbonyl ethylene; βME – β-mercaptoethanol; GLY – glycine; DMSO – dimethylsufoxide.

Materials and Methods

Materials

1-(5-Nitro-2-furyl)-2-phenylsulfonyl-2-furylcarbonyl ethylene (FE1); 1-(5-phenylsulfonyl-2-furyl)–2-phenylsulfonyl-2-furylcarbonyl ethylene (FE2); and 1-(5-phenylsulfonyl-2-furyl)-2-phenylsulfonyl-2-tienocarbonyl ethylene (FE3) were synthesized in the Department of Organic Chemistry, Slovak Technical University in Bratislava, by the Lehnert condensation method. The analytical procedures applied for the determination of the structure and the purity of the substances were described elsewhere (Špirková et al. 1991). All other chemicals used in the experiments were of analytical grade, and were purchased from Sigma (USA) and Lachema (Czech Republic).

Kinetics of reactions of furylethylene with β-mercaptoethanol (βME) and glycine (GLY)

Furylethylenes (10 μmol/l) were left to react with βME (2.5; 5; 10, and 25 μmol/l at pH 7.0) or GLY (10; 25 and 50 μmol/l at pH 9.0) in a final volume of 2.5 ml of phosphate or borate buffer solution (10 mmol/l), directly in the spectroscopic cuvette. The reactions were monitored by means of an 8452A Hewlett Packard diode array UV spectrophotometer (USA). The kinetic characteristics of the reactions of FE1, FE2 and FE3 with βME were computed from the respective changes in absorbtion spectra occurring during the reaction, using equation (1):

$$\Delta A = \Delta A_{\infty}(1 - e^{-kt}) \tag{1}$$

Where: ΔA and ΔA_{∞} represent the differences in the absorbances of the reaction mixture between the start of the reaction and at time t or $t \rightarrow \infty$ respectively. Measurements were performed in that region of the UV spectra in which the changes were most expressed. The equilibrium parameters ΔA_{∞} were plotted as a function of the bulk concentrations of βME or GLY. Maximal changes of absorbance $\Delta \underline{A}_{\infty}$ were extrapolated from the plateau phase of the latter curves. Assuming that the reactions observed follow equimolar stoichiometry, the dissociation constant K_d of the respective reaction product could be computed from equation (2)

$$K_d = [(\Delta \underline{A}_{\infty} C_{\beta\text{me(gly)}} - \Delta A_{\infty} C_{\text{fe}}) \cdot (\Delta \underline{A}_{\infty} - \Delta A_{\infty})]/\Delta \underline{A}_{\infty} \cdot \Delta A_{\infty} \tag{2}$$

Where: $C_{\beta\text{me(gly)}}$ and C_{fe} represent bulk concentrations of βME (or GLY) and furylethylene respectively.

Preparation of (Na/K)-ATPase

(Na/K)-ATPase from dog kidney medulla was prepared by the method of Jorgensen (1988) using centrifugation with the zonal rotor of a UP65 Ultracentrifuge (Germany). The purity of the preparation was checked by means of the Fast system SDS electrophoresis (Pharmacia Sweden) on 8–24% gradient polyacrylamide gels as well as by estimation of the specific activity of (Na/K)-ATPase. The resulting enzyme preparation exhibited only two electrophoretically distinguished protein bands ($M_1 \sim 110$ and $M_2 \sim 35$ kDa), which corresponded to the α and β subunits of the (Na/K)-ATPase. The specific activity of the ATPase preparation was 10–25 μmol P_i/min. mg protein.

Estimation of (Na/K)-ATPase activity

(Na/K)-ATPase activity was determined as the difference between the amounts of orthophosphate liberated by splitting of ATP (0.125–4.000 mmol/l) in the presence of NaCl (1.25–100.0 mmol/l), KCl (0.125–10.000 mmol/l) and $MgCl_2$ (4 mmol/l),

and in the presence of 4 mmol/l of $MgCl_2$ only. Enzyme reaction was running for 10 min. at 37°C, in 0.5 ml of 50 mmol/l imidazole-HCl buffer, pH 7.0 in the presence of 1–2 mg of enzyme protein. It was started by simultaneous addition of different concentrations of ATP and furylethylenes, and stopped by ice-cold trichloroacetic acid (12%). Furylethylenes were dissolved in DMSO: the final concentration of the solvent in the reaction medium did not exceed 0.5% (v/v). The liberated orthophosphate was estimated by means of the method of Taussky and Shorr (1953). The protein content in the membrane fractions was determined according to Markwell et al. (1978). Parameters of the enzyme kinetics were obtained by non-linear regression of the initial reaction velocities (V) as a function of both the concentration of ATP or cation (c) and the concentration of the inhibitor (c_i), using the PC software Sigma Plot 5.0. Computations were based on the Michaelis-Menten relationship equipped with the Hill cooperativity constant (n) and inhibition constants K_{in} and K_{ic} for both, the non-competitive and the competitive mode of inhibition respectively (Eq. 3).

$$V = [V_{max}/(1 + c_i/K_{in})] \cdot c^{n(1-k c_i)}/[K_{0.5}/(1 + c_i/K_{ic})]^{n(1-k c_i)} + c^{n(1-k c_i)} \quad (3)$$

Where: V_{max} represents the initial velocity of the enzyme reaction at concentrations of ATP (sodium or potassium) $c \to \infty$; $K_{0.5}$ represents the affinity constant for ATP (sodium or potassium), and k is a constant characterizing the dependence of the cooperativity constant on the inhibitor concentration (assuming that this dependence is linear). Apparent kinetic constants (V_{max}^{App}, $K_{0.5}^{App}$ and n^{App}) i. e. the constant obtained for the inhibitor concentration c_i, are given by the following equations:

$$V_{max}^{App} = V_{max}/(1 + c_i/K_{in}) \quad (4)$$

$$K_{0.5}^{App} = K_{0.5}/(1 + c_i/K_{ic}) \quad (5)$$

$$n^{App} = n \cdot (1 - k \cdot c_i) \quad (6)$$

Results

Comparison between (Na/K)-ATPase inhibition by furylethylenes and their reactivity with nucleophilic substances

Substance FE1 was found to react with βME when the latter was applied as a model nucleophilic substance. The reaction led to flattening or disappearance of two out of three characteristic absorption maxima of FE1 (at 256 and 360 nm). The third absorption maximum (at 305 nm) remained unaltered (Fig. 1, panels *A*,

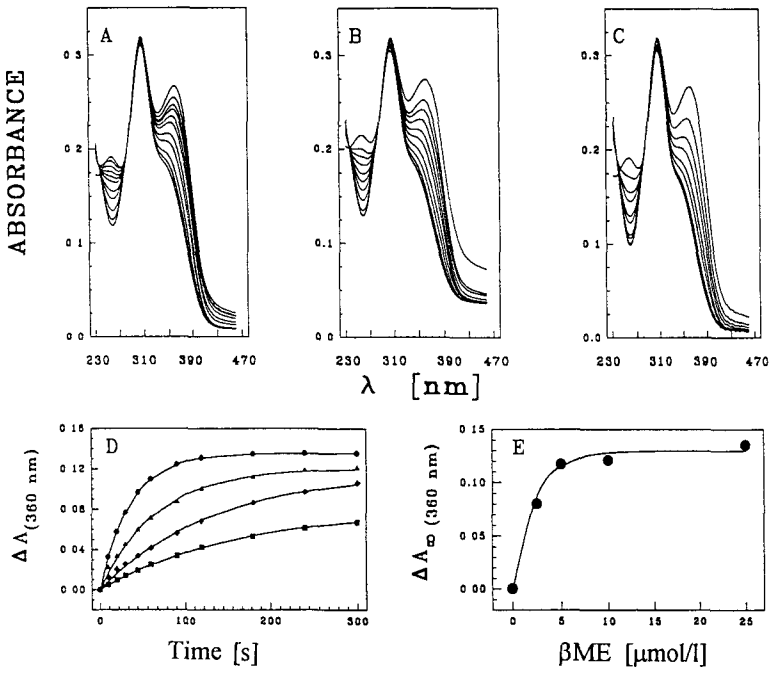


Figure 1. Kinetics dependences of FE1 reaction with βME. Furylethylenes (10 μmol/l) were left to react with βME (in μmol/l) (5 – panel *A*, 10 – panel *B* and 25 panel *C*) in a final volume of 2.5 ml of phosphate buffer solution (10 mmol/l, pH 7,0). Time dependences of the absorbance changes in FE1 (maximum at 360 nm) due to reaction with βME were fitted according to equation 1, and are shown in panel *D*. The equilibrium parameters ΔA_∞ obtained by nonlinear regression were plotted as a function of the bulk concentrations of βME (panel *E*), and maximum changes of absorbance $\Delta \underline{A}_\infty$ were extrapolated from the plateau phase of this plot. Values from six independent measurements. S.E.M. never exceeded 5% of the mean values.

B, *C*). Being most expressed, the changes in absorbance at 360 nm were used for the assessment of the kinetics of the FE1 – βME reaction (Fig. 1, panel D). Fitting the experimental data to equation (1), a good agreement with the computed data could be obtained. The kinetic parameters obtained by non-linear regression are shown in Table 1. As already indicated in Materials and Methods, the values of $\Delta \underline{A}_\infty$ were determined by extrapolation from the plateau phase of the relationship of ΔA_∞ to βME concentration (Fig. 1*E*), and the dissociation constant of the product between the reaction of FE1 and βME given in Table 2 was calculated from equation (2), applying the respective values of ΔA_∞ and $\Delta \underline{A}_\infty$. Also, the dissociation constants of the reaction of other furylethylenes with βME or GLY, which are given in Table 2, were estimated similarly. From Table 2 it also follows

Table 1. FE1 reaction with βME: parameters of kinetics dependence

βME [μmol/l]	ΔA_∞	k [s^{-1}]	$\Delta \underline{A}_\infty$
2.5	0.080 ± 0.006	0.00621 ± 0.00092	
5.0	0.117 ± 0.009	0.00748 ± 0.00103	
10.0	0.130 ± 0.012	0.01508 ± 0.00174	
25.0	0.135 ± 0.014	0.02773 ± 0.00248	
			0.14 ± 0.02

ΔA_∞ and k were obtained by nonlinear regression of experimental data from Fig. 1. according to equation 1, and represent computed value ± S.E.M. (for 8 degrees of freedom). $\Delta \underline{A}_\infty$ was obtained by extrapolation from the plateau phase of the plot in Fig. 1 *E*.

Table 2. Dissociation constants characterizing the product of reaction between furylethylenes, and βME and GLY

Furylethylene	Disociation constant βME [μmol/l]	GLY [μmol/l]
FE1	0.423 ± 0.153	19.378 ± 5.765
FE2	24.323 ± 9.835	-*
FE3	27.351 ± 9.538	-*

Data represent mean ± S.E.M. from 4 independent values. *There were no observable changes in UV spectra during 20 minutes of incubation of the respective reactants.

that furylethylenes react with a higher affinity with βME than with GLY. Judging upon the dissociation constants in the Table 2, among all the furylethylenes tested it was FE1 which showed the highest reactivity towards nucleophilic groups. Correspondingly, the latter compound was also the only one among the substances tested capable of inhibiting (Na/K)-ATPase activity to a considerable extent (Fig. 2). The concentration range of 0–20 μmol/l used in the testing of the inhibitory effects of furylethylenes was determined experimentally (not shown), and its upper limit was given by the maximum of water solubility of the compounds tested.

The effect of FE1 on (Na/K)-ATPase activation by potassium ions

Using equation (3) for fitting of the kinetics of activation of (Na/K)-ATPase activity by potassium in the presence of different concentrations of FE1, the constant K_m reached unrealistically high values. For this reason, the member $(1 + c_i/K_m)$ in equation (3) could be omitted in later computations (Fig. 3, Table 3). This indicated

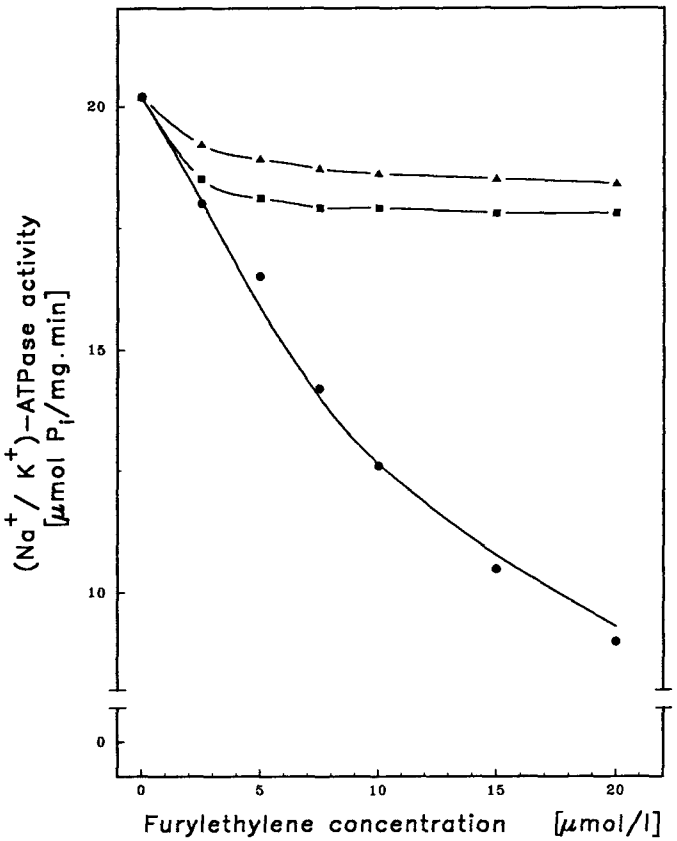


Figure 2. Inhibition of (Na/K)-ATPase activity by FE1 (circles), FE2 (squares), and FE3 (triangles). The enzyme reaction was started by simultaneous addition of ATP (4 mmol/l) and furylethylenes (0–20 μmol/l) and stopped by ice-cold trichloroacetic acid (12%) added to the reaction mixture (see Materials and Methods). Furylethylenes were dissolved in DMSO. The final concentration of the solvent in the reaction medium did not exceed 0.5% (v/v). Values from six independent measurements. S.E.M. never exceeded 5% of the mean values.

that the apparent V_{max}^{App} value is independent of the inhibitor concentration (Fig. 6 panel *A*). On the other hand, the inevitability of constants k and K_{ic} for the description of the enzyme kinetics by means of equation (3) indicates that increasing concentrations of FE1 gradually decrease the apparent cooperativity constant n^{App} and increase the apparent $K_{0.5}^{App}$ for potassium. Hence, investigation of the kinetics of inhibition of (Na/K)-ATPase activity by FE1, with respect to the mechanism of activation of the enzyme by increasing concentrations of potassium, revealed a competitive type of interaction.

Table 3. Kinetic parameters of (Na/K)-ATPase inhibition by FE1

	FE1-induced inhibition of (Na/K)-ATPase with respect to		
	Potassium ions	Sodium ions	ATP
V_{max} [μmol P_i/min.mg]	21.72 ± 0.56	25.18 ± 0.58	27.58 ± 0.47
K_m [μmol/l]	1.58 ± 0.09	14.23 ± 0.71	0.71 ± 0.03
K_{ic} [μmol/l]	3.56 ± 0.19	*	*
K_{in} [μmol/l]	*	13.31 ± 0.73	29.49 ± 2.81
n	1.49 ± 0.08	1.40 ± 0.07	*
k	0.045 ± 0.005	*	*

Results were obtained by nonlinear regression of data from Figs. 3–5 using equation (3). Results are expressed as computed values ± S.E.M. for degree of freedom in the range 32–36. * – indicates that the respective constant was not necessary for a successful fitt.

The effect of FE1 on (Na/K)-ATPase activation by sodium

In contrast to results obtained for potassium, when using equation (3) for fitting of the kinetics of activation of (Na/K)-ATPase activity by sodium, in the presence of different concentrations of FE1, it was the constant K_{ic} the value of which became unrealistically high, and thus the member $(1 + c_i/K_{ic})$ could be omitted (Fig. 4, Table 3). Moreover, the constant k reached values close to zero. This indicates that both the apparent n^{App} and the apparent $K_{0.5}^{App}$ for sodium are independent of the inhibitor concentration (Fig. 6, panel B). The inevitability of the constant K_{in} for the description of the enzyme kinetics points to a gradual decrease of apparent V_{max}^{App} induced by increasing concentrations of FE1. All this indicates that FE1 interferes non-competitively with (Na/K)-ATPase activation by sodium ions.

The effect of FE1 on the ATPase reaction kinetics

In contrast to the activation of the enzyme with potassium and sodium ions, the activation of (Na/K)-ATPase with ATP exhibits classical non-cooperative kinetics. For this reason in the fitting procedure the exponents $n \cdot (1 - k\ c_i)$ in equation (3) were set as 1. Constant K_{ic} became unrealistically high also in this case (Fig. 5, Table 3), indicating that the apparent $K_{0.5}^{App}$ for ATP is independent of the concentration of FE1. On the contrary, the value of apparent V_{max}^{App} decreases with the increasing concentration of the inhibitor applied (Fig. 6, panel C), i.e. the type of interaction may be classified as non-competitive.

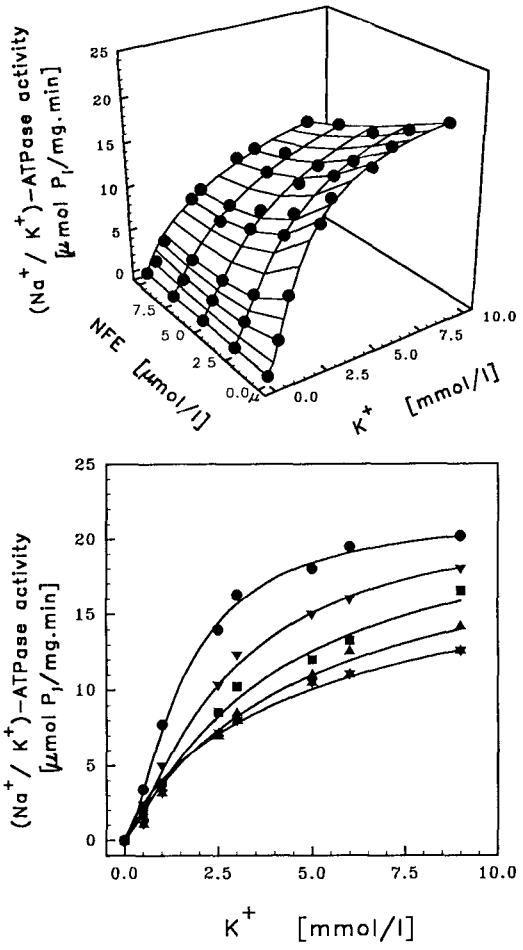


Figure 3. Effect of FE1 on the stimulation of (Na/K)-ATPase activity by potassium ions. Experimental data were fitted according to equation (3) by nonlinear regression, and the respective parameters are shown in Table 3. Upper panel: Three-dimensional plot of (Na/K)-ATPase activities versus potassium and FE1 concentrations as two independent variables. Lower panel: Two-dimensional plots of (Na/K)-ATPase activities observed in the presence of FE1 (in μmol/l : 0 – circles, 2.5 – triangles down, 5 – squares, 7.5 – triangles up, and 10 – asterisks) versus potassium concentration. Values from six independent measurements. S.E.M. never exceeded 5% of the mean values.

Discussion

In our previous study (Vrbanová et al. 1994; Breier et al. 1995), FE1 was found to stimulate the mechanical activity of isolated perfused guinea pig heart. This effect was linked with competitive inhibition of activation of the sarcolemmal (Na/K)-ATPase by potassium ions. It was assumed that the latter inhibition was a result of the interaction of the electrophilic FE1 with some nucleophilic amino acid residue located inside, or in the vicinity of the potassium binding site of the enzyme molecule. However, the partially purified fraction of heart sarcolemmal membranes used in the above study did not allow a detailed kinetic analysis of the inhibitory effect of FE1 on the (Na/K)-ATPase. Therefore, with the aim to check their gen-

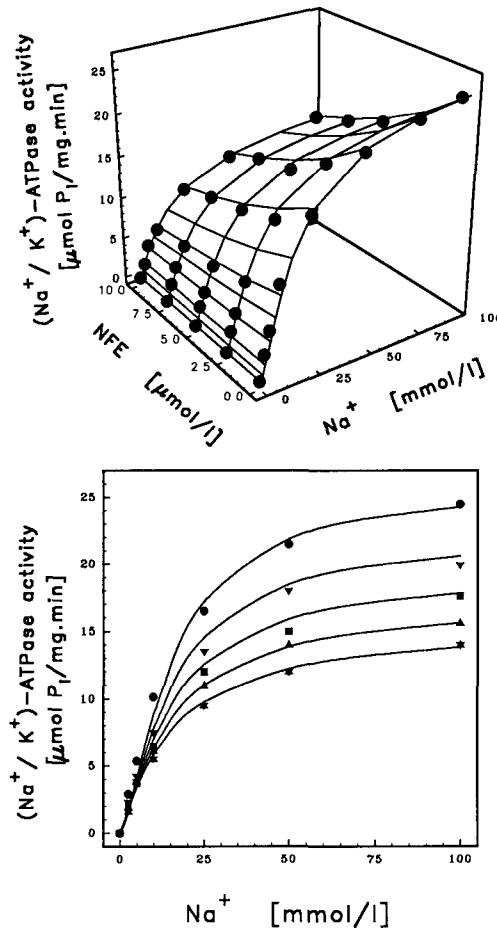


Figure 4. Effect of FE1 on the stimulation of (Na/K)-ATPase activity by sodium ions. Experimental data were fitted according to equation (3) by nonlinear regression, and the respective parameters are shown in Table 3. Upper panel: Three-dimensional plot of (Na/K)-ATPase activities versus sodium and FE1 concentrations as two independent variables. Lower panel: Two-dimensional plots of (Na/K)-ATPase activities observed in the presence of FE1 (in μmol/l: 0 – circles, 2.5 – triangles down, 5 – squares, 7.5 – triangles up, and 10 – asterisks) versus sodium concentration. Values from six independent measurements. S.E.M. never exceeded 5% of the mean values.

eral validity, highly purified (Na/K)-ATPase preparation from dog kidney medulla (Jorgensen 1988) was used in the present study to verify our earlier data about the mechanism and character of the inhibitory action of FE1.

The structure of FE1 contains two electrophilic moieties (Fig. 7) which are able to react with nucleophilic groups of proteins: i.) a conjugated triene moiety (I) characterized by delocalization of a π-electron due to the presence of an electron-attracting NO_2 group in position on the furylethylene skeleton, ii.) a formyl group (II) which connects the second furyl ring with the furylethylene moiety (Špirková et al. 1991). In FE2 and FE3 the reactivity of the conjugated triene moiety (I) was reduced due to replacement of the NO_2 group with a weaker electron-attracting phenylsulfonyl group. The reactivity of the formyl group was lowered by replacing the second furyl ring (in FE1 and FE2) with the more aromatic thienyl ring (in

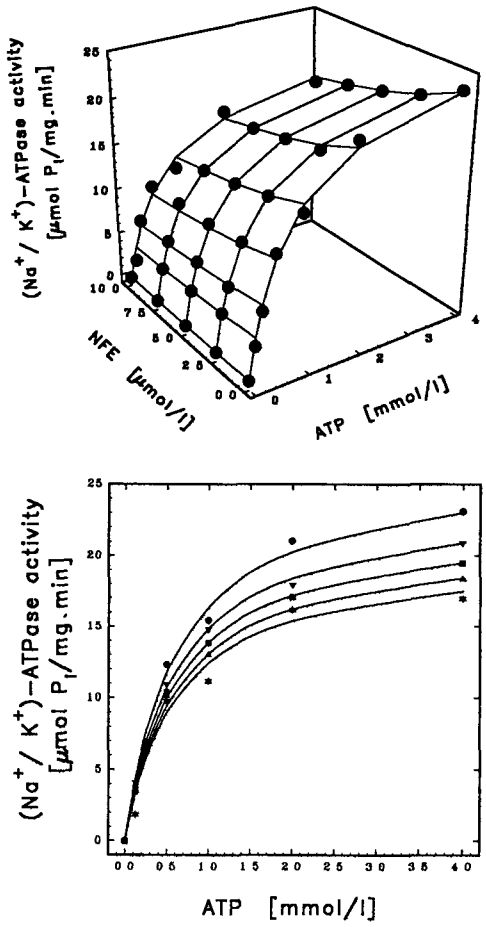


Figure 5. Effect of FE1 on the stimulation of (Na/K)–ATPase activity by ATP Experimental data were fitted according to equation (3) by nonlinear regression, and the respective parameters are shown in Table 3 Upper panel Three-dimensional plot of (Na/K)-ATPase activities versus ATP and FE1 concentrations as two independent variables Lower panel Two-dimensional plots of (Na/K)-ATPase activities observed in the presence of FE1 (in μmol/l 0 circles, 2 5 triangles down, 5 squares, 7 5 triangles up, and 10 – asterisks) versus ATP concentration Values from six independent measurements S E M never exceeded 5% of the mean values

FE3, Fig 7) Our finding that among the compounds tested, FE1 exhibited the highest affinity to nucleophilic substances (Fig 1, Tables 1 and 2) points to the conjugated triene moiety as the structure which may be responsible for the reactivity with nucleophilic compounds Sulfhydryl groups may be considered to be more potent nucleophils than the amino or hydroxyl groups (Drobnica and Gemeiner 1976, Drobnica et al 1977, Gemeiner and Drobnica 1979) This explains the higher reactivity of the electrophilic conjugated triene moiety with the SH-group of βME in comparison to that of the NH_2 group of GLY (Table 2) The described model experiments offer an explanation also for the molecular mechanism of the reported inhibition of (Na/K)-ATPase by FE1 (Fig 2) The interaction of the electrophilic conjugated triene moiety of FE1 with a nucleophilic group of an amino acid residue

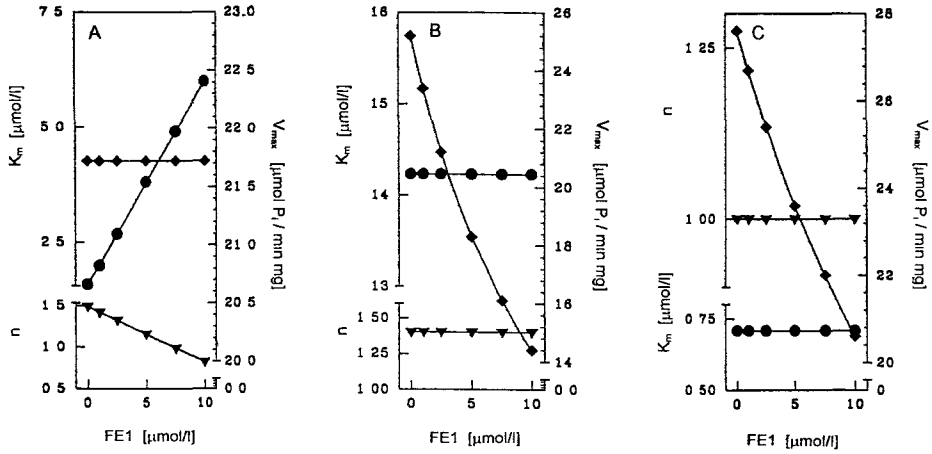
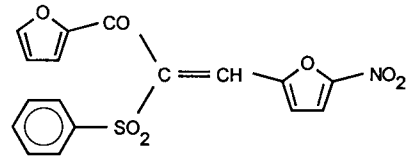


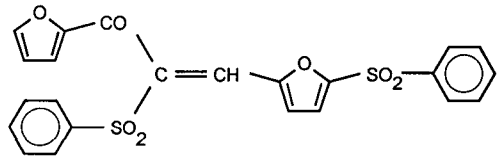
Figure 6. Dependence of the kinetic constants (V_{max}^{App} – diamonds, $K_{0.5}^{App}$ – circles and n^{App} – triangles) on FE1 concentration for (Na/K)-ATPase activity stimulation by· potassium ions – panel *A*; sodium ions – panel *B* and ATP – panel *C* The kinetic constants were computed according to equations (4) – (6) using data from Table 3.

located somewhere in the (Na/K)-ATPase molecule may be considered responsible for the above phenomenon.

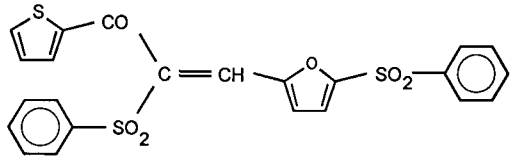
The assumption that FE1 interacts competitively with (Na/K)-ATPase in the potassium binding site of the enzyme is based on our findings that, due to the action of FE1, the affinity of the enzyme to potassium and the cooperativity between its potassium binding sites are depressed i.e., n^{App} is reduced, the apparent $K_{0.5}^{App}$ is increased, and the V_{max}^{App} for the activation with potassium remains unchanged (Figs. 3 and 6, Table 3). There seems to be much similarity between the effects of furylethylenes on (Na/K)-ATPase and that of the selective amino reagent 2,4,6-trinitrobenzenesulfonic acid which also depressed the stimulation of the enzyme by potassium ions (De Pont et al. 1984; Breier et al. 1986, 1987). Nevertheless, neither the latter studies nor the present investigation give a definitive answer about the character of the functional group of the interacting amino acid residue on the enzyme. This is because furylethylenes are able to interact with the amino group and with sulfhydryl and hydroxyl groups as well (Rosenberg et al. 1985; Baláž et al. 1987). Moreover, in the present study we have shown that FE1 interacts more strongly with the SH group of βME than with the NH_2 group of GLY (Table 2). Therefore, it should be admitted that not only the NH_2 group, as suggested earlier (Breier et al. 1986), but also other nucleophilic groups, such as the SH or OH group, may be of essential importance for the binding of potassium to (Na/K)-ATPase. This is in good agreement with our previously published hypothesis about the role



1-(5-NITRO-2-FURYL)-2-PHENYLSULFONYL-2-FURYLCARBONYL ETHYLENE (FE1)



1-(5-PHENYLSULFONYL-2-FURYL)-2-PHENYLSULFONYL-2-FURYLCARBONYL ETHYLENE (FE2)



1-(5-PHENYLSULFONYL-2-FURYL)-2-PHENYLSULFONYL-2-TIENOCARBONYL ETHYLENE (FE3)

Figure 7. Structure of the furylethylenes tested. I conjugated triene moiety of the furylethylene skeleton, II – formyl group that connects the second furyl ring (FE1, FE2) or thienyl ring (FE3) to the furylethylene moiety.

of nucleophilic amino acid residues in the process of formation of complex bounds of potassium in potassium binding sites of the (Na/K)-ATPase (Ziegelhöffer et al. 1986; Breier et al. 1988).

In contrast to activation by potassium ions, FE1 inhibited the activation of (Na/K)-ATPase by sodium ions non-competitively only (Figs. 4 and 6 panel *B*, Table 3). In this respect, the effect of FE1 proved to be similar to that observed with 2,4,6-trinitrobenzene sulfonic acid (Breier et al. 1986) i.e. the substance has no effect on the apparent $K_{0.5}^{App}$ for the activation of the enzyme by sodium.

With respect to the locus and the molecular mechanism of interaction between

FE1 and (Na/K)-ATPase it can be assumed that the substance may chemically modify the enzyme at one of two different specific sites: i) either directly inside or in the vicinity of its potassium binding sites, or ii) inside or in the vicinity of its ouabain binding sites which are in regulatory relationship to (Na/K)-ATPase activation by potassium ions (Skou and Essman 1992). As concerns the possibility sub ii) this appears highly improbable from the following reasons: if FE1 were bound at the ouabain binding site, its relation to ouabain would be competitive. Accordingly, it would inhibit the activation of (Na/K)-ATPase by potassium ions, inducing similar structural changes in the (Na/K)-ATPase molecule as does ouabain. However, the missing structural similarity between FE1 and ouabain speaks against such a possibility. On the other hand, structural similarity itself cannot be classified as a determining prerequisite for similarity in action. The structurally different aspartic acid has been shown to not only depress the potassium activation of (Na/K)-ATPase in an ouabain-like manner (similar reaction kinetics), but to even compete with ouabain for the same binding site on the enzyme molecule (Fedelešová et al. 1975). Nevertheless, ouabain and aspartic acid were found to depress the potassium-mediated activation of (Na/K)–ATPase allosterically (Lulman et al. 1975; Hansen 1984) whereas FE1 does it in a competitive way. This suggests that FE1 and ouabain may bind to different loci of (Na/K)-ATPase. Hence, only the possibility sounds realistic that FE1 interacts at the potassium binding site of (Na/K)-ATPase. This assumption is based on the capability of FE1 to interact with some of the available nucleophilic groups (probably amino groups) located inside or close to the potassium binding sites, and thus to compete with potassium for the binding to the enzyme (Figs. 3 and 6 panel *C*, Table 3). An essential role of amino groups in the binding of cations to proteins has recently been demonstrated also on the example of the calcium binding sites of calreticulin (Breier and Michalak 1994). In this case, a chemical modification of the respective nucleophilic amino acid residues with the electrophilic 2,4,6-trinitrobenzenesulfonic acid resulted in competitive inhibition of calcium binding to this protein.

Isothiocyanates were shown to inhibit competitively the binding of ATP to (Na/K)-ATPase. This is a result of the chemical modification of an essential NH_2 (Carilli et al. 1982) or SH residues (Ziegelhöffer et al. 1983; Breier et al. 1989) at the ATP binding site of the enzyme. However, as a strong electrophilic substance which is also able to react with these groups, FE1 does not exhibit any competitive action against binding of ATP to the (Na/K)-ATPase (Figs. 5 and 6 panel *A*, Table 3). The diversity between the effects of isothiocyanates and FE1 on (Na/K)-ATPase points to further differences in chemical properties of the nucleophilic groups involved in the binding of ATP and potassium to their respective specific binding sites. Further detailed investigations will be still necessary to further elucidate the nature of these differences.

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Final version accepted July 20, 1996