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Inhibition of Na,K-ATPase by a new ATP analog, adenosine-5-*N'*-(2,4-dinitro-5-fluorophenyl)phosphohydrazide

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A new ATP analog, adenosine-5-*N'*-(2,4-dinitro-5-fluorophenyl) phosphohydrazide (DNPH-AMP), has been synthesized, which is an irreversible inhibitor of Na,K-ATPase. Interaction of the analog with the enzyme in the presence of K^+ is described by the scheme:

$$E + I \underset{k_2}{\overset{k_1}{\leftrightarrow}} E \cdot I \xrightarrow{k_3} E - I,$$

and corresponding kinetic constants k_3 and K_i are found equal to 2.5 min^{-1} and 1.6 mM. In the presence of Na^+ the time course of enzyme inactivation by DNPH-AMP is a biphasic curve in the semilogarithmic plot. The k_3 and K_i values calculated for this case according to Fritzsch [Fritzsch (1985) J. Theor. biol. 117 397] are equal to 2.45 min^{-1} and 2.5 mM, respectively. ATP transforms the K^+-type of Na,K-ATPase inactivation into the one that takes place in the presence of Na^+.

Na,K-ATPase; ATP analog; Irreversible inhibition

1. INTRODUCTION

Na,K-ATPase hydrolyzes ATP supporting active transport of Na^+ and K^+ across the outer cell membrane. During the catalytic cycle the enzyme undergoes conformational transition from E_1 to E_2 state. At the beginning of the cycle Na,K-ATPase exists in E_1-conformation which has a high affinity to Na^+ and ATP. E_2-conformation being formed in the second part of the cycle has lower affinity to these ligands but high affinity to K^+ [1–3]. Therefore the interaction of Na^+ and/or ATP with the enzyme shifts the equilibrium between E_2 and E_1 forms toward E_1, whereas K^+ induces transformation of the Na,K-ATPase molecule into the E_2 state [2,4–6].

There is a number of indications that Na,K-ATPase contains nucleotide-binding sites with high and low affinity to ATP [5–7]. The structure of these sites remains unknown. The replacement of natural substrates by their analogs, which inactivate the enzyme by covalent binding (affinity labelling) is a commonly used approach for the study of the enzymes' active centres. We describe here the kinetics of inactivation of E_1- and E_2-conformations of Na,K-ATPase by the new ATP analog DNPH-AMP, which can modify SH-, OH- or NH_2-groups.

Abbreviations: DNPH-AMP, adenosine-5-*N'*-(2,4-dinitro-5-fluorophenyl) phosphohydrazide; MOPS, *N*-morpholinopropanesulfonic acid; NBS^{6}ITP, 6-[(3-carboxy-4-nitrophenyl)thiol]-9-β-D-ribifuranosylpurine triphosphate

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2. MATERIALS AND METHODS

ATP ('Reanal') was recrystallized according to Berger [8] and converted into the imidazole salt by passage over Dowex-50 in the H-form with subsequent neutralization with imidazole. MOPS was obtained from Sigma, sucrose from Fluka. Other chemicals (reagent grade) were obtained from Reachim, USSR.

DNPH-AMP was synthesized by the following method. 2,4-Dinitro-1,5-difluorobenzene (245 mg) was dissolved in 4 ml of dioxane then 124 mg adenosine-5-phosphohydrazide (lithium salt) and 80 mg lithium acetate in 4 ml H_2O were added (pH 8.0 was maintained by addition of 1 N LiOH). The mixture was incubated with stirring overnight in the dark at 20°C and then evaporated to a volume of 1 ml and centrifuged; the supernatant was mixed with 5 ml of anhydrous methanol and 5 ml of diethyl ether. The precipitate was separated, washed by anhydrous ether and dried in vacuo. The resulting powder was dissolved in 2 ml of H_2O, then 10 ml of anhydrous methanol and 3 ml of diethyl ether were added. The precipitate was separated and dried in vacuo. DNPH-AMP was converted into the imidazole salt by ion-exchange chromatography.

Na,K-ATPase from duck salt glands was prepared according to the method of Hopkins et al. [9]. The specific activity was usually in the range of 25–30 μmol/mg protein/min. Mg-dependent ATPase was absent in these preparations.

Inactivation of the enzyme by DNPH-AMP was performed by incubation of 1.4–2 mg protein in a total volume of 1 ml in 150 mM NaCl (or KCl), 3 mM $MgCl_2$, 0.5–4 mM DNPH-AMP and 30 mM MOPS (pH 7.5) at 20°C. In some experiments 1–3 mM ATP was also present. After 0.5–15 min incubation 5–10 μl aliquots were withdrawn from the medium and Na,K-ATPase activity was determined.

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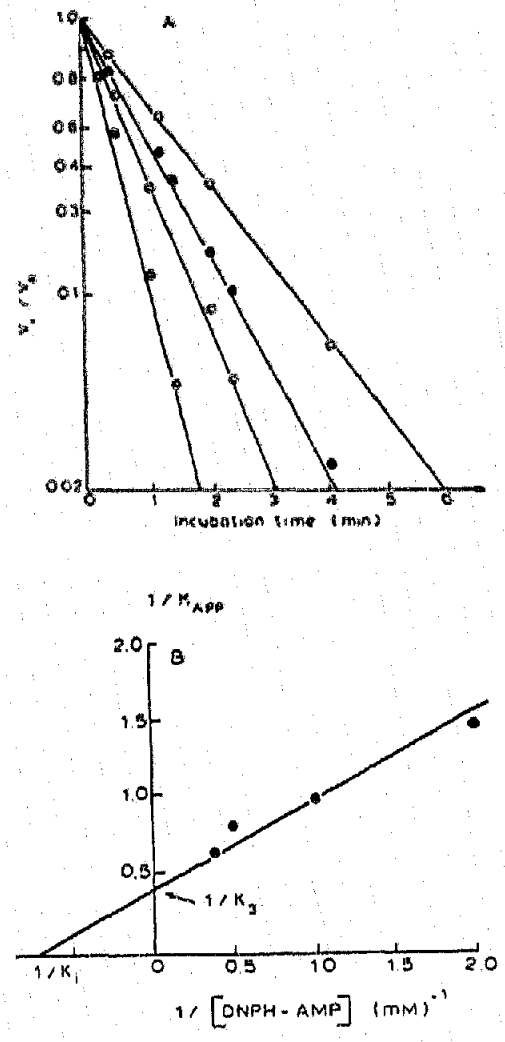

Fig. 1. Inactivation of Na,K-ATPase by DPNH-AMP in the presence of 150 mM KCl. (A) Dependence of residual activity (V_i/V_0) on preincubation time in semilogarithmic coordinates. DNPH-AMP concentrations: 0.5 mM (○), 1 mM (●), 2 mM (⊙), 3 mM (⊖). (B) Dependence of apparent inhibition constants, K_{APP}, calculated from (A), on DNPH-AMP concentration (double reciprocal coordinates).

Activity of Na,K-ATPase was measured at 37°C in 1 ml of solution containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP and 30 mM imidazole (pH 7.5). The reaction was stopped by addition of 1 ml of 3 M acetic buffer and liberated P_i was determined [10].

3. RESULTS AND DISCUSSION

The interaction of Na,K-ATPase with DNPH-AMP occurs slowly and depends on both analog concentration and time of preincubation (Fig. 1A). Transferring of the enzyme into the medium for assay of ATPase activity results in 50-200-fold dilution of the DNPH-AMP. Nevertheless the enzyme activity was not restored. This reflects the irreversible character of Na,K-ATPase inhibition by the analog.

When inactivation of the enzyme with different DNPH-AMP concentrations was performed in the presence of K^+, a family of straight lines was obtained in a semilogarithmic plot (log V_i/V_0 vs time), indicating the pseudo-first order kinetics of inactivation (Fig. 1A). If the apparent inactivation rate constant (k_{app}) calculated from the slopes of the curves on Fig. 1A is plotted versus DNPH-AMP concentration, a hyperbolic curve is obtained. It can be linearized in a double-reciprocal plot (Fig. 1B). This type of inhibition was described previously for substrate-like inhibitors by the following scheme [11]:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} E \cdot I \xrightarrow{k_3} E\text{-}I \qquad (1)$$

where E·I and E-I are enzyme-inhibitor complexes, in which the inhibitor is bound to the enzyme in reversible and irreversible manner, respectively. The k_3 and K_i values calculated for the interaction of DNPH-AMP with K-conformation (E_2-form) of the enzyme according to this scheme are presented in Table I.

Table I

Kinetic constants for inactivation of the Na- and K-conformations of Na,K-ATPase by DNPH-AMP

Constants	Na-conformation	K-conformation
K_i, mM	3.50	1.6
K_3, min^{-1}	2.45	2.5
K_4, min^{-1}	0.435	
K_5, min^{-1}	0.023	
$K_3/K_3 + K_4$	0.050	

In the presence of Na^+ the time course of enzyme inactivation is biphasic, being represented in the semilogarithmic plot by two linear branches (Fig. 2). The time of transition from one branch to another (~2 min) does not depend on the inhibitor concentration.

This type of inactivation of Na,K- and Ca-ATPase by ATP analogs was described recently [12-14] and theoretically analyzed by Fritzsch and Koepsell [15].

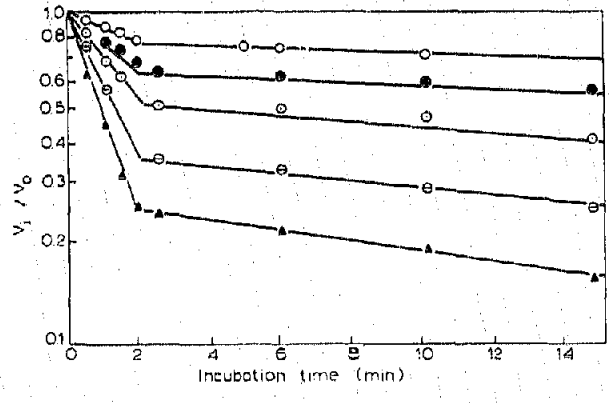

Fig. 2. Inactivation of Na,K-ATPase by DNPH-AMP in the presence of 150 mM NaCl. DNPH-AMP concentrations 0.5 mM (○), 1 mM (●), 2 mM (⊙), 3 mM (⊖), 4 mM (▲).

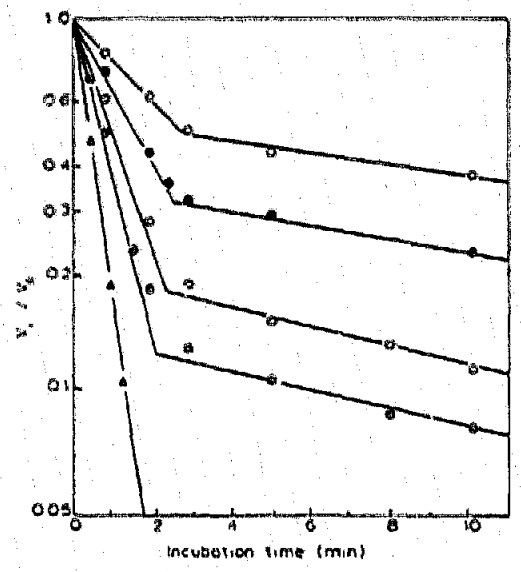

Fig. 3. Effect of ATP on Na,K-ATPase inactivation by DNPH-AMP in the presence of 150 mM KCl. DNPH-AMP concentrations: without ATP, 3 mM (▲), in the presence of 1 mM ATP, 1 mM (○), 2 mM (●), 3 mM (◎), 4 mM (⊖).

According to the model proposed by these authors the enzyme can exist in two slowly interconverting forms, E and E*, only one of which (E) is accessible for the analog:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} E \cdot I \xrightarrow{k_3} E\text{-}I \qquad E \underset{k_5}{\overset{k_4}{\rightleftarrows}} E^* \qquad (2)$$

The simple procedure for calculation of k_4 and k_5 was described by Fritzsch [16]. It was also shown that constant $K = k_2/k_1$ under quasi-stationary conditions is close to K_i. The value of K_i in this case could be found from the values of k_3, k_4 and from the transition time on the biphasic curve. The values of kinetic constants for the interaction of DNPH-AMP with Na-conformation (E_1-form) of the enzyme obtained with the use of this approach are presented in Table I.

As seen from Table I, the K_i and k_3 values for the interaction of analog with E_1- and E_2-conformations are quite similar. This implies that DNPH-AMP interacts with the same ATP-binding site both in the presence of Na^+ or K^+. The only difference is that in the presence of Na^+ the amount of the conformation accessible for inhibitor is rather small, being equal to 5% of the total amount of the enzyme (according to the equation $E = k_5/(k_5 + k_4)$ [16]).

Addition of ATP (1–3 mM) into K^+-containing medium results in a decrease of the rate of enzyme inactivation. Moreover, at the simultaneous presence of ATP and K^+ the time course of the inactivation becomes a biphasic one (Fig. 3). These findings support the data in accordance to which not only Na^+, but also ATP can converts Na,K-ATPase into the Na-conformation [2,4,6].

The interaction of DNPH-AMP with both E_1- and E_2-forms of the enzyme is characterized by the low affinity (K_i values are in millimolar range of analog concentration). Sufficiently high K_i value (1.4 mM) was also found for the inactivation of Na,K-ATPase by another ATP analog: Nbs^6ITP [16]. These values are much higher than K_d for ATP binding with the high-affinity site (0.2–2 μM) [5] and are suppos ; to reflect binding with a low-affinity binding site.

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REFERENCES

[1] Jorgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399–415.
[2] Karlish, S.J.D. and Yates, D.W. (1978) Biochim. Biophys. Acta 527, 115–130.
[3] Skou, J.C. (1988) in: Methods in Enzymology, vol. 156 (S. Fleischer and B. Fleischer eds) pp. 1–25.
[4] Skou, J.C. (1982) Biochim. Biophys. Acta 688, 369–380.
[5] Ottolenghi, P.L. and Jensen, J. (1983) Biochim. Biophys. Acta 727, 89–100.
[6] Lopina, O.D., Pindel, E.V. and Boldyrev, A.A. (1990) FEBS Lett. 266, 75–77.
[7] Gache, C., Rossi, B. and Lazdunski, M. (1976) Eur. J. Biochem. 65, 293–306.
[8] Berger, L. (1956) Biochim. Biophys. Acta 29, 23–26.
[9] Hopkins, B.E., Wagner, H.J. and Smith, T.W. (1976) J. Biol. Chem. 251, 4365–4371.
[10] Rathbun, W. and Betlach, M. (1969) Anal. Biochem. 28, 436–445.
[11] Kitz, R. and Wilson, J.B. (1962) J. Biol. Chem. 237, 3245–3249.
[12] Koepsell, H., Hulla, W. and Fritzsch, G. (1982) J. Biol. Chem. 257, 10733–10741.
[13] Patzelt-Wenzler, R. and Schoner, W. (1981) Eur. J. Biochem. 114, 79–87.
[14] Pick, U. (1981) Eur. J. Biochem. 121, 187–195.
[15] Fritzsch, G. and Koepsell, H. (1983) J. Theor. Biol. 102, 469–476.
[16] Fritzsch, G. (1985) J. Theor. Biol. 117, 397–415.