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Effect of *N*-ethylmaleimide on ($Na^+ + K^+$)-dependent adenosine triphosphatase activity at very low substrate concentration

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N-ETHYLMALEIMIDE (NEM) inhibits ($Na^+ + K^+$)-dependent adenosine triphosphatase [($Na^+ + K^+$)-ATPase] activity[1] without affecting an Na^+-dependent ATP-[^{14}C]ADP transphosphorylation believed to be the initial step of the reaction.[2,3] The authors assumed a sequence of two enzyme-phosphorylated intermediates in the ($Na^+ + K^+$)-dependent reaction, which for the purposes of this communication is simplified as follows:

$$ATP + E_1 \underset{}{\overset{Mg^{2+}\ Na^+}{\rightleftharpoons}} ADP + E_1 \sim P \qquad (1)$$

$$E_1 \sim P \xrightarrow{Mg^{2+}} E_2 - P \qquad (2)$$

$$E_2 - P \xrightarrow{K^+} E + P_i \qquad (3)$$

Several distinctive properties between both phosphorylated intermediates have been described later.[4] It is assumed that NEM inhibits step (2) of the reaction.[2,4]

An Na^+-dependent and K^+-independent ATPase (Na^+-ATPase) activity has been described at very low substrate concentrations in preparations with ($Na^+ + K^+$)-ATPase activity. These two activities were either attributed to different enzymatic sites acting independently,[5] or to a single enzyme or enzyme system.[6]

This paper aims at describing the effect of NEM on Na^+-ATPase activity, in order to provide additional material for the solution of this problem. As yet we have found no report of such study in the literature.

The preparation of rat brain microsomes and the test of Na^+- and ($Na^+ + K^+$)-ATPase activities are reported elsewhere.[6] It was previously shown[6] that the results obtained in the presence of ($Na^+ + K^+$) at low ATP concentrations reveal the ($Na^+ + K^+$)-dependent ATPase activity of the preparation and not a combination of Na^+- and ($Na^+ + K^+$)-dependent ATPase activities. [γ-^{32}P]ATP, prepared according to Glynn and Chapell,[7] was used as substrate. All other reagents were analytical grade. Prior to enzymatic activity tests, the microsomes were preincubated at 37° with different NEM concentrations for different periods.

Two different groups of experiments were performed and typical results are summarized in Table 1.

For ATP concentrations higher than 1 mM, the inhibition of ($Na^+ + K^+$)-ATPase by NEM required preincubation of the enzyme with the drug.[1] The first group of experiments showed that preincubation is also required at concentrations under 1 μM ATP. In addition, the data showed that Na^+-ATPase activity was also inhibited by NEM and that the inhibition of both Na^+- and ($Na^+ + K^+$)-ATPase activities varied similarly with different preincubation periods.

TABLE 1. EFFECT OF NEM on $(Na^+ + K^+)$-ATPASE ACTIVITY AT VERY LOW SUBSTRATE CONCENTRATIONS*

	Preincubation (min)	[NEM] (mM)	ATPase activity (μmole of P_i/mg of protein × hr) Additions: Mg^{2+}	$Mg^{2+} + Na^+$	$Mg^{2+} + Na^+ + K^+$
Experiment 1	0	1·0	0·33	1·54	1·63
[ATP], 0·66 μM	5	1·0	0·22	0·89	1·04
[Enzyme], 7·6 μg of protein/ml	10	1·0	0·26	0·45	0·36
Experiment 2	5	10·0	0·20	0·15	0·18
[ATP], 0·55 μM	5	3·0	0·30	0·72	0·69
[Enzyme], 5·6 μg of protein/ml	5	1·0	0·54	1·25	1·39
	5	0·3	0·50	1·47	1·69
	5	0·1	0·52	1·82	1·81
	5		0·57	1·77	1·82

* Microsomes were preincubated in the presence of NEM, 30 mM Tris buffer (pH 7·4) and cations as specified in the Table. For ATPase activity tests, the reactions were started by adding (γ-^{32}P)ATP immediately after preincubation, and stopped with 5% final concentration of trichloroacetic acid, 2 min later. Chloride salts of the cations were used at the following concentrations: 6 mM $MgCl_2$, 100 mM NaCl and 20 mM KCl.

The effects of different NEM concentrations during 5-min preincubations were tested in the second group of experiments. No differences were detected between the inhibitory effects of NEM on Na^+- and $(Na^+ + K^+)$-ATPase activities. Thus, different preincubation times and NEM concentrations similarly increased the inhibitory effect of the drug on both types of ATPase activity.

Previous results had led us to think of two different mechanisms of the same enzyme or enzyme system, with or without K^+.[6] The similar effect of NEM on both activities reinforces this hypothesis and further suggests that both activities are affected by NEM at a common step of the reaction.

Thus, it is necessary to evaluate why K^+ fails to activate ATP hydrolysis at low substrate concentrations. This presumably occurs when reaction (1) is rate limiting, which is also the case when uridine 5′-triphosphate (UTP) replaces ATP as substrate.[8] Siegel and Goodwin[8] assumed an equilibrium between two enzyme forms with different affinities for Na^+ and K^+. Reaction (1) admits the enzyme form with high Na^+ affinity. In the presence of K^+, a reduction of this enzyme form would take place.

Fahn *et al.*[2] proposed that NEM inhibits $(Na^+ + K^+)$-ATPase activity by preventing the transformation of $E_1 \sim P$ into $E_2 - P$, without blocking Na^+-dependent transphosphorylation and formation of $E_1 \sim P$. With this hypothesis in mind, our results suggest that Na^+-dependent ATPase activity reflects the hydrolysis of $E_2 - P$ in the absence of K^+, since this type of enzymatic activity is also inhibited by NEM.

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