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Research article

The effect of EGTA and Ca^{++} in regulation of the brain Na/K-ATPase by noradrenaline

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Abstract

Background: The Na/K-ATPase activity of the brain synaptic plasma membranes (SPM) is regulated by noradrenaline (NA) and the synaptosomal factor SF (soluble protein obtained from the synaptosome cytosol). In the absence of SF, NA inhibits Na/K-ATPase, while, on addition of SF to the reaction medium, there is a NA-dependent activation of Na/K-ATPase . On the other hand, EGTA augments the Na/K-ATPase activity and attenuates the ability of NA to inhibit Na/K-ATPase.

Results: Considering that Ca^{2+} ion is a Na/K-ATPase modifier, it can be assumed that the effect of NA and SF is a Ca^{2+}-dependent process. However, in the presence of 0.3 mM EGTA and 0.1 mM NA, the apparent inhibition constant for Ca^{2+} (at $[Ca^{2+}] > 0.3$ mM) is not SF dependent, while the apparent activation constant for SF does not change at increasing Ca^{2+} concentration ($[Ca^{2+}] < 0.3$ mM). At various Ca^{2+} concentrations (0.06, 0.35 and 0.6 mM), no significant changes occur in the mode of action of NA on the Na/K-ATPase activity in the presence of 5 μg/ml SF. EGTA also has no effect on the NA-independent activation of Na/K-ATPase evoked by high SF concentrations.

Conclusions: Taking into account that in the absence of EGTA similar results have been obtained, it can be concluded that the effect of NA and SF on brain Na/K-ATPase is a Ca^{2+}-independent process.

Background

Noradrenaline (NA) and some other neurotransmitters (acetylcholine, dopamine and serotonin) are involved in the regulation of the Na/K-ATPase activity of the synaptic plasma membranes. In the presence of the synaptosomal factor SF (soluble protein obtained from the synaptosome cytosol) NA activates, while in the absence of SF inhibits Na/K-ATPase [1–4]. The effect of neurotransmitters on Na/K-ATPase is of strictly specific character and, is apparently related to the regulation of synaptic transmission. However, the mechanism and functional significance of the effect of NA/SF on the Na/K-ATPase system has not been fully ascertained. There arise many questions and one of them is the problem of participation of Ca^{2+} ions [5,6]. Ca^{2+} ion and EGTA are modifiers of the Na/K-ATPase system and at the same time EGTA sharply attenuates the inhibition of Na/K-ATPase by NA [2]. Therefore arises a necessity to clarify the role of Ca^{2+} ions in the mechanism of action of NA and SF on the Na/K-ATPase system.

Results and Discussion

It should be especially emphasized that EGTA, NA and NA together with SF (NA-dependent activation) have no effect on ouabain-insensitive Mg-ATPase. On the other hand, EGTA causes an increase of the Na/K-ATPase activity, in spite of washing of the Na/K-ATPase preparations in the solutions containing chelators of bivalent ions [6]. Such an action of EGTA cannot be explained only as a consequence of binding of endogenous ions of Ca^{2+}, and apparently, is also a result of a direct effect of EGTA on Na/K-ATPase [7,8]. In our preparations at low concentrations (up to 0.1 mM), EGTA increases the Na/K-ATPase activity, which remains unchanged up to 0.6 mM EGTA. Simultaneously, in the presence of EGTA NA looses its ability to inhibit Na/K-ATPase, while in the absence of NA in the reaction medium, no statistically significant change occurs with SF (5–80 μg/ml) on Na/K-ATPase .

It has been earlier demonstrated [9] that in the absence of EGTA the dependence of inverse value of the Na/K-ATPase activity on the concentration of Ca^{2+} ions (10^{-5}–10^{-3} M) has a strictly linear character and the apparent inhibition constant is $K_i = 0.30 \pm 0.01$. However, in the presence of 0.3 mM EGTA in the reaction medium, the dependence has a non-linear character (Fig. 1). At low concentrations of Ca^{2+} ions (<0.3 mM) non-linear dependence is observed, while at high concentrations of Ca^{2+} ions (>0.3 mM), the dependence appears to have a linear character and the apparent constant of inhibition is $K_i = 0.072 \pm 0.010$ (Table 1). The broken line in fig. 1 shows an inverse value of the Na/K-ATPase activity in the absence of EGTA and $[Ca^{2+}]$. Apparently, the decrease in K_i due to the presence of EGTA, as well as the increase of the Na/K-ATPase activity at low concentrations of Ca^{2+} ions may partially be accounted for by binding of free ions of Ca^{2+} by EGTA.

It has been earlier demonstrated that in the absence of EGTA, addition of NA and SF into reaction medium does not alter the Ca^{2+} apparent inhibition constant [9]. This enabled us to conclude that the effect of NA and SF on the Na/K-ATPase system is not a Ca^{2+}-dependent process. On the other hand, EGTA is supposed to act directly on the Na,K-ATPase system [7,8]. It has been also demonstrated that EGTA alters the stoichiometry of binding of Na^+/K^+/ATP and the basic kinetic parameters of dependence of the Na/K-ATPase activity on MgATP, free ATP and Mg^{2+}.

Consequently, in order to assert firmly that the effect of NA and SF on the Na/K-ATPase system is not Ca^{2+} – independent, it is necessary the enzyme to be studied in the presence of EGTA in the reaction medium. To this end, 0.3 mM EGTA was chosen, because of its saturation effect. As it is shown in table 2 there are changes in the (%) Na/K-ATPase activity, produced by adding various concentrations of NA in the presence of 0.3 mM EGTA, 5 μM/ml SF and three different concentrations of Ca^{2+} ions. I – 0.06 mM $[Ca^{2+}]$, which causes an activation of Na/K-ATPase ; II – 0.35 mM $[Ca^{2+}]$, at which the activity becomes equal to the original level; III – 0.6 mM $[Ca^{2+}]$, eliciting a sharp inhibition; (see Fig. 1). As seen in table 2, at various concentrations of Ca^{2+} ions no statistically significant changes occur in the Na/K-ATPase activity, expressed in % (in all cases $P > 0.1$).

Fig. 2 shows the dependence of the Na/K-ATPase activity (inverse values) on the concentration of Ca^{2+} ions, in the presence of 0.1 mM NA and 0.3 mM EGTA in the reaction medium. In the range 0.3 mM ≤ $[Ca^{2+}]$ ≤ 1 mM experimental points have linear dependence and were subjected to regression analysis ($1/V = A + B\,[Ca^{2+}]$; $K_i = A/B$), the results yielded are exhibited in table 1. As seen in table 1, the apparent inhibition constant K_i is not altered at various concentrations of SF, and does not differ from K_i obtained in the absence of NA and SF (in all cases $P > 0.1$).

Table 1: Dependence of Ca^{2+} apparent inhibition constant on [SF].

NA, mM	SF, μg/ml	A	B	K_i
	20	0.0042 ± 0.0013	0.0529 ± 0.0020	0.080 ± 0.025
0.1	30	0.0037 ± 0.0003	0.0449 ± 0.0005	0.082 ± 0.007
(fig. 2)	40	0.0032 ± 0.0013	0.0410 ± 0.0020	0.078 ± 0.032
	80	0.0031 ± 0.0018	0.0410 ± 0.0027	0.076 ± 0.044
		Weighted mean, K_i		0.082 ± 0.001
0. 1 (fig. 1)	0	0.0043 ± 0.0008	0.0599 ± 0.0005	0.072 ± 0.010

Table 2: Dependence of the Na/K-ATPase activity on NA with various [Ca^{2+}], in the presence of 0.3 mM EGTA and 5 μg/ml SF. The activity at [NA] = 0 is considered as 100% (Number of identical measurements is 6)

NA,	The Na/K-ATPase activity (%)		
mM	[Ca^{2+}] = 0.06 mM	[Ca^{2+}] = 0.35 mM	[Ca^{2+}] = 0.6 mM
0.025	112.7 ± 3.1	111.9 ± 3.3	109.7 ± 2.4
0.05	106.8 ± 1.7	106.9 ± 2.6	109.7 ± 2.6
0.1	110.5 ± 4.6	108.7 ± 3.7	111.3 ± 3.4
0.5	93.2 ± 3.6	91.1 ± 2.4	90.0 ± 3.6
1	85.7 ± 2.3	86.1 ± 3.2	85.5 ± 4.1

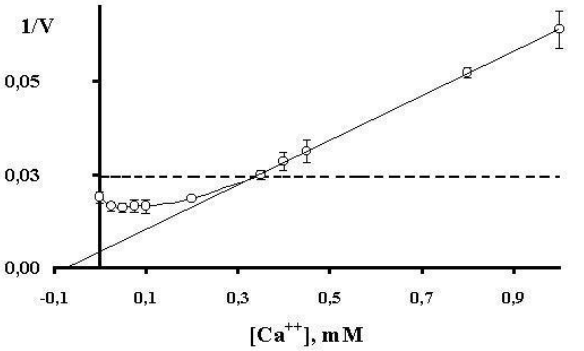

Figure 1
Dependence of inverse values of Na/K-ATPase activity (μmol P_i/hour × mg protein) on [Ca^{2+}] concentration (mM), in the presence of 0.3 mM EGTA. Broken line shows inverse value of Na/K-ATPase activity (I/V0) in the absence of EGTA and Ca^{2+} ions.

Similar kinetic analysis of a nonlinear part of curves, shown in fig. 2, is impossible. Therefore we have studied the dependence of the Na/K-ATPase activity with various concentrations of SF in the presence of 0.3 mM EGTA, 0.1 mM NA, and during variation of Ca^{2+} ion concentration (fig. 3). In inverse values this dependence has a linear character. Regression analysis yielded the following parameters, ($1/V = A + B\ [1/SF]$, $K_a = B/A$):

[Ca^{2+}] = 0; A = 0.0144 ± 0.0002; B = 0.1111 ± 0.0039; K_a= 7.72 ± 0.29

[Ca^{2+}] = 0.1 mM; A = 0.0136 ± 0.0002; B = 0.1034 ± 0.0109; K_a= 7.60 ± 0.83

Also, Ca^{2+} ions in this case do not change the apparent activation constant for SF in the presence of EGTA and SF ($P > 0.1$).

Changes in the Na/K-ATPase activity, with the addition of NA and SF into the reaction medium, represent the summated effect of several discrete processes [1]: 1) NA-dependent inhibition. (In the absence of SF, NA inhibits Na/K-ATPase); 2) NA-dependent activation, that occurs at low concentrations of SF (SF has no direct effect on the Na/K-ATPase activity); 3) NA-independent activation occurs in the lack of NA and high concentrations of SF. In the absence of EGTA all these processes appear to be Ca^{2+}-independent [9].

On the other hand, the SF elicited NA-independent enhancement of the Na/K-ATPase activity expressed in %, which remains unchanged by adding 0.3 mM EGTA, even at its high concentrations (unpublished data). In the presence of EGTA and fixed concentration of SF, Ca^{2+} ions do not change the dependence of the Na/K-ATPase activity (%) on NA (table 2). In the presence of EGTA and fixed concentration of NA we have independence of SF and Ca^{2+} action of the Na/K-ATPase activity (fig. 2,3; table 1)

The problem of Ca^{2+} ions participation in the regulation of the Na/K-ATPase activity by NA arises from the following two main reasons. First, Ca^{2+} ions play an important role in synaptic transmission. Second, in broken cell preparations, NA seems to enhance the Na/K-ATPase activity nonspecifically by chelating divalent metal ions that normally inhibit the enzyme [5]. The EGTA evoked activation of Na/K-ATPase, seems to confirm this observation. However, the existence of a direct effect of EGTA on Na/K-ATPase, as well as the independence of Ca^{2+} inhibition constant of SF concentration and other above indicated experimental findings unequivocally refute the claim.

Conclusions

Consequently, the regulation of the brain Na/K-ATPase activity by NA and SF is a Ca^{2+}-independent process. On the other hand, the effect of NA and EGTA are interdependent processes. The obtained results seem to have an important functional significance and therefore are, in need, of further study.

Materials and Methods

Materials

Sephadex G-50 and G-25 were obtained from Pharmacia (Uppsala, Sweden), bovine serum albumin (BSA) – from Reanal (Budapest, Hungary), ouabain – from Carbio-

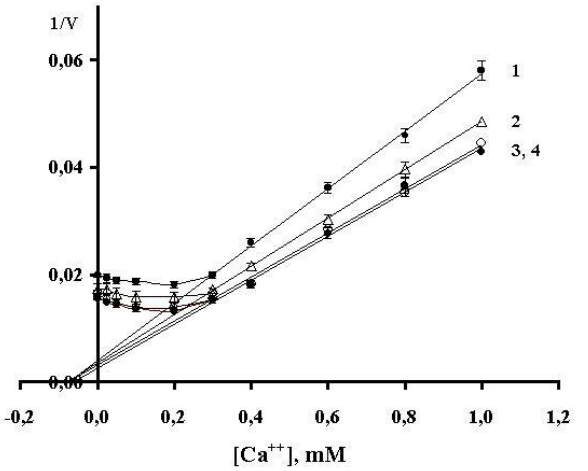

Figure 2
Dependence of inverse values Na/K-ATPase activity (μmol P_i/hour × mg protein) on [Ca^{2+}] concentration (mM), in the presence of 0.3 mM EGTA, 0.1 mM NA and at various concentrations of SF. 1 – 20 μg/ml SF; 2 – 30 μg/ml SF; 3 – 40 μg/ml SF; 4 – 80 μg/ml SF.

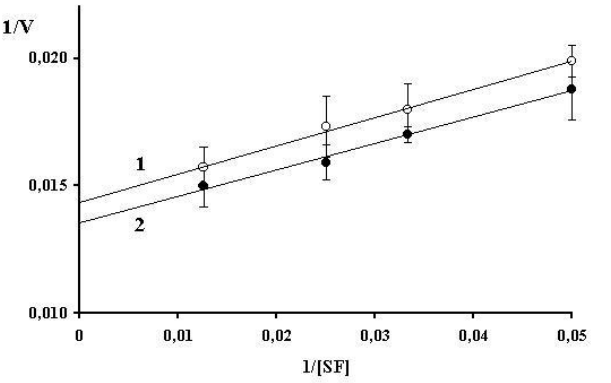

Figure 3
Dependence of Na/K-ATPase activity (μmol P_i/hour × mg protein) on SF concentration (μg/ml) in inverse values and in the presence the of 0.3 mM EGTA, 0.1 mM NA and various concentrations of the Ca^{2+}-ions. 1 – no Ca^{2+}-ions added; 2 – added [Ca^{2+}] = 0.1 mM.

chem (San Diego, California, USA) and ATP-Na_2 – from Sigma (USA).

Isolation of synaptic membranes and synaptosomal factors
With the aim to obtain synaptic membrane fractions a slightly modified classical method was used. The rats were decapitated and the brains were rapidly removed within 80 s. The brain homogenates (10% w/v) were prepared in 0.32 M sucrose solution using the mild 'manual' homogenization and a "crude mitochondria" fraction (consisting of synaptosomes) was obtained. Following the osmotic shock (9 ml H_2O per 1 g of fresh tissue), crude mitochondria fraction was centrifuged at 25000 g, for 40 min. The obtained sediment (P_2Wp) and supernatant (P_2Ws) were used for further fractionation. P_2Wp was resuspended (1.2 M sucrose, 3 mM EGTA and 3 mM EDTA) and centrifuged at 110000 g, for 75 min. After dilution of the supernatant (down to 0.9 M sucrose, 3 mM EGTA and 3 mM EDTA) and centrifugation at 110000 g, for 85 min, the sediment (P_2Wp [1.2–0.9]), consisting of synaptosomal membranes, rich in the junction complexes, was obtained. The P_2Ws supernatant was centrifuged again at 100000 g, for 30 min, and the obtained supernatant was used for extraction of the synaptosomal factors (SF).

Further fractionation factor was made on the chromatograph (LKB, Sweden), on 2 × 20 and 2 × 40 columns filled with G-50 diluted in 5 mM of Tris-HCl buffer (pH-7.3). Fractionation produced two peaks – the first, relatively high-molecular (SF, 60–100 kD); the second, the low-molecular one (≅ 10 kD). From the first eluate (peak 1) SF was concentrated by Sephadex G-25.

Before their utilization, the preparations of synaptic membranes and synaptosomal factors were stored at < [-20°C] for two months (without changes of enzyme activity and modifying effect).

Evaluation of the ATPase activity
Na/K-ATPase was measured as an ouabain-sensitive part of the total ATPase, while an ouabain-insensitive part was presented by Mg-ATPase. The total ATPase reagent medium contained 2 mM ATP, 2 mM $MgCl_2$, 140 mM NaCl, 5 mM KCL, and 50 mM Tris-HCl buffer, at pH 7.7. Mg-ATPase was determined by adding 0.2 mM ouabain into the medium consisting of 145 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, and 50 mM Tris-HCl buffer, at pH 7.7. At these total concentrations of ATP and $MgCl_2$ it can be asserted with more confidence that there is no substrate saturation, high sensitivity of the enzyme to bivalent cations and little probability of the formation of CaATP cmplex [8]. Reaction conditions and activity measurements were standard, as it was described earlier [4].

The data on the Na/K-ATPase and Mg-ATPase activities are presented in μmol P_i /mg protein × hour and are assigned as V, or are expressed in percent (V,%). Phosphorus was measured by the modified Fiske and Subbarow method [11] and protein concentration – according to the Lowry method [12]. The BSA served as the standard.

Statistical analyses

The data are presented as mean ± s.e.m. The number of identical measurements was from four to eight. Relative error was mostly unchanged and fluctuated between 2.5% and 10%. Experiments were often repeated three-four times and their unification was made by weighted averaging method. The curves were expressed by polynominal approximation, the straight lines – by regression analysis.

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