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ENDOGENOUS FACTOR ACTIVATING Na,K-ATPase INDUCED BY BLOCKADE OF ADRENOCEPTORS

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Abstract—It was established that the regulatory factor released from the effector cells under the blockade of postsynaptic α- and β-adrenoceptors by phentolamine and propranolol (0.5–1.0 μM) and activating noradrenergic uptake and synthesis [1, 2] increased the activity of Na,K-ATPase of isolated rat organs and partially purified Na,K-ATPase isolated from the rat brain. The regulatory factor is a protein with molecular mass 25–100 kDa, adsorbed on phenylsepharose CL-4B and eluated with 30 mM KCl. It consists of two active components with pI 5.1 and 5.9 which in admixture (1:1) activate Na,K-ATPase by 60%.

Previously, a peripheric mechanism was found for the reverse transsynaptic regulation of catecholamine uptake and synthesis in adrenergic neurons, in which the initial links are postsynaptic adrenoceptors of the effector cell. Blocking these receptors leads to the formation and release from the effector cell of chemical regulatory factors. The latter reach the presynaptic membrane through the synaptic gap and produce an increase in the rate of transport into the adrenergic neuron of noradrenaline and its major precursor amino acid tyrosine [1, 2].

It is known that blockers of Na,K-ATPase influence the uptake of noradrenaline [3]. Thus, ouabain inhibits noradrenaline uptake [4] and enhances its efflux [5], and noradrenaline activates Na,K-ATPase [6]. The effect of noradrenaline could not be the result of direct effect of the enzyme, but rather mediated by membrane lipids [7]. Therefore, action of the adrenergic system should be closely related to the activity of Na,K-ATPase.

These facts suggest that the main target of the action of regulatory factors may be Na,K-ATPase of the presynaptic membrane of adrenergic neurons. The work presented here was undertaken to test the idea by studying biochemical properties of the regulatory factor formed under the effect of adrenoblockers and its effect on the activity of Na,K-ATPase.

MATERIALS AND METHODS

The experiments were performed on male white Wistar rats, weighing 180–200 g. The animals were decapitated, and the vas deferens (80–90 mg) and atrium (60–80 mg) were excised and incubated in Tyrode solution (NaCl 120 mM; KCl 20 mM; $MgCl_2$ 2.5 mM; pH 7.8) or homogenized in this solution.

To obtain the medium containing the regulatory factor the isolated organs were incubated in Tyrode solution in the presence of adrenoblocking agents (phentolamine or propranolol, 1–5 μM) for 60 min [1, 2].

Study of the effect of the regulatory factor on ATPase activity in the extracts of isolated organs. The vas deferens and atria were homogenized (5 mg w/w per 1 mL of the medium), the homogenates were centrifuged (500 *g*, 20 min), and the supernatant was used in the experiments. In some experiments homogenate was made in the medium previously used for incubation of the isolated organs with adrenoblocking agents. In the study of the direct effect of adrenoblocking agents on the extracts these agents were preincubated with the extracts for 15 min. To assay ATPase activity 50 μL of 500 mM Tris buffer (pH 7.4) and up to 2 mM ATP were added to 500 μL of extract and incubated for 20 min at 40°. The reaction was stopped by adding 50 μL of 50% trichloroacetic acid (TCA). Inorganic phosphate (P_i) was determined according to Lowry and Lopez [8]. The measurements were made on a Specol-21 spectrophotometer.

Assay of ATPase activity of the surface structures of isolated organs. Adrenoblocking agents (phentolamine or propranolol) were introduced into a temperature-controlled bath with the isolated organs after 30-min preincubation, then ATP was added to 0.2 mM or the organs were transferred to the tubes with 3 mL of incubation medium of the same composition and ATP was added to 0.2 mM. In either case incubation was continued for another 40 min at 37° under oxygenation after which the formation of inorganic phosphate was determined.

Assay of ATPase activity by measuring H^+ formation. Concentration of H^+ ions was measured on a "Radiometr A/C" autotitrator (Denmark) at pH 7.4 maintained by 0.01 M NaOH. The isolated organs were placed into a temperature-controlled (37°) chamber of the apparatus, incubated for 30 min and ATPase activity was measured twice, the background activity and that after the addition of ATP to 0.2 mM, for 10 min in either case. The difference between the two measurements was taken as a characteristic of ATP activity. The organs were then washed for 5 min and placed into the medium containing a blocking agent of adrenoceptors or in the medium where other isolated organs had been

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incubated with adrenoblocking agents. In the first case preincubation with an adrenoblocking agent continued for 30 min after which the activity of ATPase was registered. In the second case there was no preincubation before registering the activity.

Effect of the regulatory factor on Na,K-*ATPase.* To obtain Na,K-ATPase, the brains of five rats were homogenized in a Potter homogenizer for 3 min at 700 rpm in 70 mL of the following medium: 0.25 M sucrose, 50 mM Tris buffer (pH 7.4), 4.5 mM EDTA and 0.1% heparin. The homogenate was centrifuged for 15 min at 10,000 *g*, and a two-layer precipitate was formed. The supernatant was kept for further use and the upper layer was carefully washed off and then homogenized again in 70 mL of the same medium at 1500 rpm for 5 min, after which it was centrifuged for 15 min at 10,000 *g*. The supernatant obtained was centrifuged together with the first one for 45 min at 45,000 *g*. The precipitate was resuspended in 50% glycerol and then kept at −20°. In this preparation Na,K-ATPase activity was 30% of the total ATPase activity. To detect the regulatory factor, 20 μL of 500 mM Tris buffer (pH 7.4), 5 μL of 20 mM ATP and 10 μL of ATPase preparation were added to 200 μL of the medium where isolated organs had been incubated with adrenoblocking agents and incubated for 30 min at 40°. In this case the enzyme activity was assayed from the formation of inorganic phosphate.

Study of the biochemical properties of the regulatory factor. To obtain the regulatory factor with the use of pharmacological drugs the isolated organs were incubated for 1 hr in the following solution; 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 50 mM Tris–HCl, pH 7.68 (solution A) supplied with 5 μM phentolamine or propranolol. For ultrafiltration of the protein solutions an FM-001 chamber and Millipore filters (U.S.A.) with the rejection masses 10, 25 and 100 kDa were used. Ultrafiltration was performed at 4° and 4×10^5 Pa nitrogen pressure. The molecular mass of the proteins was determined by electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate (SDS) [9].

Analytical focusing was performed in polyacrylamide gel, containing 4% of acrylamide, 0.0012% methylene-bisacrylamide, 10% manite and 2% ampholines. The gel was 0.5 mm thick and 0.05 N H_3PO_4 and 0.05 N KOH were used as electrode solutions.

For isofocusing in a wide pH range a 2% solution of ampholines pH 3.5–10 was used. For isofocusing in the pH range 4–7 we used the mixture of ampholines of the following composition: one part pH 3.5–10, four parts pH 4–6, four parts pH 5–7.

For isoelectric focusing of proteins in micropreparative amounts granulated gel Sefadex G-50 (Pharmacia Fine Chemicals, Sweden) was used. Upon the completion of the procedure the gel was collected in tubes in bands 5 mm thick, and the proteins were eluated with 2 mL 50 mM Tris–HCl buffer, pH 7.6.

The effect of the isolated fractions was determined from a change in Na,K-ATPase activity.

The protein was determined according to Lowry *et al.* [10]. The data obtained were statistically processed using the Student's criterion.

The drugs used were propranolol, ouabain, ATP, SDS, Coomassie R-250 (Serva, F.R.G.), phentolamine (Fluka, Switzerland), NaCl, KCl, $MgCl_2$, Tris, trichloroacetic acid, NaOH, sucrose, EDTA, heparin, H_2PO_4, mannitol, 199 medium (Khimreaktiv, USSR), Tris–HCl, pH 7.68 ("Ultrol", Calbiochem-Behring Corp., Sweden), acrylamide, methylene-bisacrylamide (Reanal, Hungary), phenyl-sepharose CL-4B, Sephadex G-50, ampholines (Pharmacia Fine Chemicals, Sweden).

RESULTS

It was shown earlier that under the blockade of postsynaptic adrenoceptors there takes place activation of noradrenaline uptake and synthesis in nerve endings, and blockade of the protein synthesis removes this effect [1, 2]. As the transport processes are energy dependent, we proposed that there should take place a change in the activity of Na,K-ATPase under these conditions.

Potentiometric determination of ATPase activity

We found no direct effect of phentolamine or propranolol (0.1–1.0 μM) on ATPase activity of vas deferens, atrium or their extracts. Neither were there found any changes in the enzyme activity in the period from 2 to 20 min after the addition of the blocking agents and ATP to the incubation medium.

The increase in the activity of ATPase under the effect of phentolamine and propranolol was usually found no earlier than 25–35 min after the beginning of incubation of isolated organs with ATP and adrenoblocking agents and persisted for an additional 30 min.

In the absence of exogenous ATP the rate of acidification of the incubation medium was not affected either by adrenoblocking agents or oubain.

In the following experiments the isolated organs were pretreated for 30 min with the adrenoblocking agents without ATP. Then after the addition of ATP the rate of its hydrolysis by vas deferens and atria was registered using the method of potentiometric titration. In these experiments 30-min blockade of α-adrenoceptors by phentolamine (1 μM) and of β-adrenoceptors by propranolol (0.5 μM) produced a significant increase of ATPase activity in the vas deferens; by 40% and 25% as compared to the starting activity. In the atria incubated with propranolol (1 μM) ATPase activity was increased by 26% (Table 1).

In order to prove that the change of ATPase activity was produced by the effect of the regulatory factor and to reveal the latter in the incubation medium the isolated vas deferens were incubated for 30 min with phentolamine or propranolol, after which the intact vas deferens with the background ATPase activity determined before the procedure were placed in this incubation medium. In these conditions ATPase activity was also significantly increased; by 45% in the experiments with phentolamine, by 30% in those with propranolol. A specific inhibitor of Na,K-ATPase ouabain (5 mM) completely eliminated activation of ATP hydrolysis.

For the study of organ specificity of the regulatory factor the vas deferens were incubated with

Table 1. Effect of regulatory factor on ATPase activity of isolated rat organs

Source of RF*	Blockers (μM)	Test-object	ATPase activity (μM P_i/mg/min)	
			Control	Effect of RF
Vas deferens	Phentolamine 1.0	Vas deferens 1	0.171 ± 0.020	0.234 ± 0.010
		Vas deferens 2	0.139 ± 0.015	0.201 ± 0.021
		Atria	0.132 ± 0.011	0.171 ± 0.008
	Propranolol 0.5	Vas deferens 1	0.162 ± 0.010	0.203 ± 0.011
		Vas deferens 2	0.184 ± 0.012	0.239 ± 0.009
Atrium	Propranolol 1.0	Atrium	0.262 ± 0.011	0.331 ± 0.018

* RF, regulatory factor.

Table 2. Effect of regulatory factor on ATPase activity

Source of RF†	Blockers (μM)	ATPase activity (μM P_i/mg/min)	
		Action of adrenoblockers	Action of RF
Vas deferens	Control	16.0 ± 0.6	18.4 ± 2.2
	Phentolamine 0.5	16.0 ± 1.2	23.6 ± 2.4*
	Phentolamine 1.2	15.6 ± 0.6	26.4 ± 1.4*
Atrium	Control	15.2 ± 0.8	16.0 ± 0.8
	Propranolol 0.5	15.0 ± 1.2	23.8 ± 1.2*
	Propranolol 1.0	15.4 ± 0.6	22.0 ± 1.2*

Difference from control is statistically significant: *$P < 0.01$.
† RF, regulatory factor.

Table 3. Effect of regulatory factor on Na,K-ATPase and Mg-ATPase

Condition	ATPase activity (μM P_i/mg/min)		
	Total	Mg-ATPase (ouabain 1 mM)	Na,K-ATPase
Control	16.8	10.4	6.4
RF*	21.2	9.2	12.0

* RF, regulatory factor.

phentolamine for 30 min whereupon the atria were placed in this medium and after the addition of ATP the enzyme activity was registered. It showed a significant increase, by 29% (Table 1).

Biochemical determination of Na,K-*ATPase activity*

The direct evidence for the effect of the regulatory factor on Na,K-ATPase activity was obtained in the experiments with the partially purified enzyme isolated from the rat brain. The blocking agents (phentolamine and propranolol) *per se* produced no changes in the enzyme activity. Upon the incubation of Na,K-ATPase in the medium containing the regulatory factor there was a significant increase of the enzyme activity. The factor produced in the vas deferens under the effect of phentolamine in the concentration 0.5 and 1 μM activated the enzyme by 28 and 43%. With the regulatory factor obtained under the effect of propranolol on the atria the enzyme was activated by 49% at the blocker concentration 0.5 μM and by 36% at 1 μM (Table 2).

The changes in the enzyme activity observed under the effect of the regulatory factor are the result of its effect precisely on Na,K-ATPase. Inhibition of Na,K-ATPase activity by ouabain (1.0 mM) completely eliminates the increase of ATPase producing no significant changes in the activity of Mg-ATPase (Table 3).

Determination of molecular mass of the regulatory factor

The best way for determining molecular mass of the regulatory factor is ultrafiltration. The advantage of this method is that the substance under study is not diluted but even becomes concentrated in the procedure. The medium containing the factor was concentrated from 10 to 1 mL. The solution that passed through the filter was collected to study the factor activity, and the concentrated solution was diluted with the standard saline solution to the original volume and repeatedly ultrafiltrated to bring its volume to 1 mL. This concentrated solution was diluted with a standard saline solution to bring its volume to 10 mL, the activity of the factor was then determined. The results of these experiments are given in Table 4. It can be seen that the regulatory factor has molecular mass in the range 25–100 kDa.

Table 4. Activity of Na,K-ATPase in U × 10^{-3} upon the effect of incubation medium fractions obtained by ultrafiltration

	Range of molecular masses (kDa) in the fractions of incubation medium			
Control	$<10^4$	10^4–2.6×10^4	2.6×10^4–10^5	$>10^5$
4.0	3.6	3.8	6.1	3.8

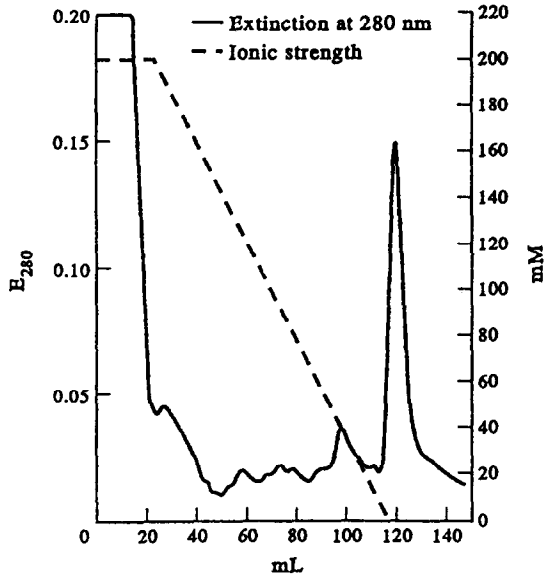

Fig. 1. Eluation of incubation media proteins from phenyl-sepharose by a linear gradient of ionic strength from 200 to 0 mM KCl.

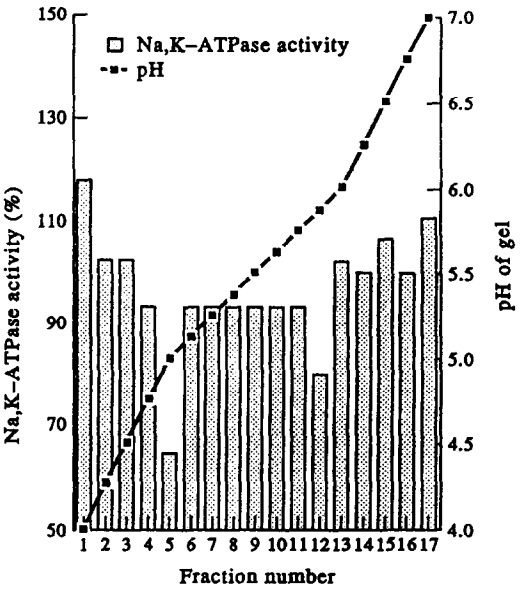

Fig. 2. Localization of regulatory factor activity after isofocusing in the pH 4–7 range.

Table 5. Amount of protein and regulatory factor activity in the fractions obtained by phenyl-sepharose chromatography of incubation media

Eluent	Amount of protein (mg)	Na,K-ATPase activity (% of control)
Unbinding protein	4205 ± 673	98
250 mM KCl	2060 ± 215	102
30 mM KCl	1.6 ± 1.2	154
Distilled water	564 ± 93	96
1% solution of Triton X-100	1272 ± 200	87

Biochemical studies of the regulatory factor

The regulatory factor for these studies was obtained by incubating the isolated organs in medium 199 with adrenoblocking agents for 18–20 hr. The incubation medium was centrifuged to separate the cells and applied onto the column with 15 mL of phenyl-sepharose CL-6B. The column was washed with solution B to bring the optical extinction E_{280} down to the original level. The proteins were eluated by a decreasing linear ionic strength gradient. To create the gradient Ultrograd (LKB), a high-ionic strength solution (solution B: 200 mM KCl and 50 mM Tris–HCl) and a low-ionic strength solution (distilled water) were used. The eluation profile of the proteins from the column with phenyl-sepharose CL-6B is presented in Fig. 1. Further on the fractions containing the regulatory factor were isolated on phenyl-sepharose CL-6B using a stepwise gradient of ionic strength.

To estimate the efficiency of the purification on phenyl-sepharose CL-6B, separate fractions eluated from the column were concentrated by ultrafiltration. Protein concentration and the effect on the activity of Na,K-ATPase were measured in these fractions, in the starting medium and in the one passed through phenyl-sepharose (Table 5). The fraction containing the regulatory factor made up 1/5000 of the total protein released in the incubation medium, and the amount of protein in it was the least stable compared to the other peaks eluated from phenyl-sepharose CL-6B.

The column was washed free of the unbound proteins with solution B, and the active fraction was eluated using solution B:water (1:10).

To perform isoelectrofocusing in the pH range 3.5–10 the medium containing the factor was concentrated by ultrafiltration. The starting volume was brought to 0.5 mL, and 50 μL of the medium were applied onto 1-mm-thick polyacrylamide gel plates containing amfolines. Upon termination of the focusing, the band with the sample was cut out, cut into 5-mm pieces along the pH gradient and

Table 6. Examination of isoelectrofocusing fractions in the pH range 4–9 for their effects on Na,K-ATPase activity

	pH									
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	8.5	9.0	Control
Activity	2.0	1.8	3.3	2.0	1.8	1.5	1.5	1.5	2.0	2.0

Table 7. Effect of regulatory factor components separated by isoelectrofocusing and their mixtures on Na,K-ATPase activity

	pI of components		Mixture
	5.1	5.9	1:1
Na,K-ATPase activity relative to control	0.83 ± 0.36	1.02 ± 0.18	1.63 ± 0.07

eluated with Tris buffer pH 7.2, 50 mM, 1 mL per piece at 4°. Parallel to this an empty band was treated in the same way, but the elution was made with distilled water in order to determine pH. The remaining gel was stained with Coomassie R-250. The eluates were tested for the ability to change Na,K-ATPase activity (Table 6). For this they were diluted 10-fold with solution A and the procedure was then the same as with the incubation medium. The eluates from the empty band served as control.

As seen from Table 6, all the activity was found in the pH range 5–6, where the main bulk of the protein was also concentrated. In the pH range 4–7 analytical focusing was performed. In parallel, the proteins from other fractions, those obtained by chromatography of the proteins of the incubation medium on phenyl-sepharose CL-6B were applied. The fraction obtained on phenyl-sepharose contained a great number of bands covering the whole pH range under study. However, the SDS gel electrophoresis of these samples demonstrated a comparatively small number of proteins, with one major protein found in the region of 65 kDa. This protein was found practically in all the fractions obtained on phenyl-sepharose CL-6B, and according to the molecular mass it corresponded to the serum albumin of rats.

The preparative separation of the fraction obtained on phenyl-sepharose and containing the regulatory factor, was performed in the granulated gel, sephadex G-50. After completion of the isofocusing the gel was separated into fractions along the pH gradient with the interval 5 mm, from which the protein was eluated with solution A, and the effect of the fractions on Na,K-ATPase was tested. A pH gradient was constructed so that in the range 5–6 there was 0.1 pH unit per 5 mm of the gel. The results of the tests are presented in Fig. 2. The fractions with isopoints 5.1 and 5.9 produced an insignificant decrease in Na,K-ATPase activity. However, because of the scattering of the results, in some of the experiments these changes were statistically

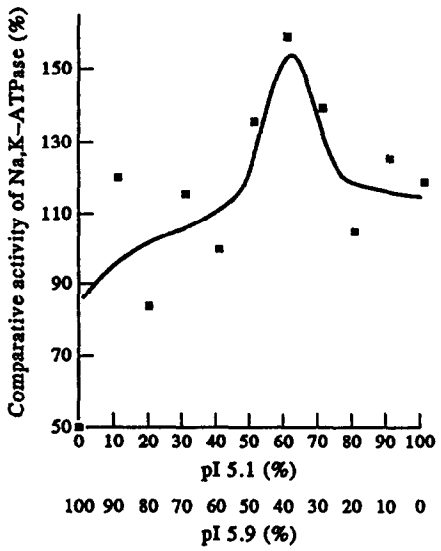

Fig. 3. Dependence of Na,K-ATPase activity on the ratio of regulatory factor components.

unreliable. These fractions mixed in a 1:1 volume ratio, gave the preparation activating Na,K-ATPase just in the same way as the starting incubation medium (Table 7). The neighbouring fractions had no such property.

The fractions with pI 5.1 and 5.9 were mixed in various ratios in such a way that concentration of one of the fractions linearly increased, and that of the other decreased from sample to sample. In Fig. 3, which shows the effect of the mixtures on Na,K-ATPase, one can see that activation of Na,K-ATPase occurs in the narrow interval of the ratios of the factor's fractions, with a pronounced maximum.

DISCUSSION

The experimental data presented here show that the blockade of postsynaptic adrenoceptors leads to the formation of the regulatory factor which, besides

enhancing the neuronal uptake and synthesis of noradrenaline [1, 2], also increases the rate of ATP hydrolysis. In the process there is an increase in the activity only of Na,K-ATPase. Its blockade by ouabain completely eliminates the activation (Table 3).

In the work of Cornish and Krstew [11] no change in the rate of neuronal uptake of [^{3}H]noradrenaline under the effect of β-adrenoblocking agents on the isolated atria of rat and cat were reported. It is impossible to explain reliably such a difference in the results of our experiments [1, 11]. Two differences in the experimental method can be noted. In our work we used a 5-mL chamber for incubating organs, while in the work of Cornish *et al.* [11, 12] they used a 2-mL chamber. In our work [1] it is pointed out that activation of neuronal uptake of noradrenaline by propranolol takes place in the narrow concentration range 0.2–1 μM. In the work of Cornish *et al.* [11] propranolol was used in concentrations 0.1, 0.5 and 5.0 μM, of which only one is effective according to our data [1]. With a small volume of the incubation chamber (2 mL), propranolol could be partially adsorbed by the atrial tissue. Its effective concentration acting on β-adrenoreceptors could, therefore, become lower than a threshold concentration and so produce no effect on the neuronal uptake. This seems to be the most probable explanation for the differences in our results. This is also confirmed by the fact that after incubation no added blocking agents (propranolol) are found spectrofluorometrically (excitation 296 nm, emission 333 nm; the minimal detectable concentration 10 nM on a MPF4 "Hitachi") in the incubation medium.

From the experimental data presented here it follows that the enzyme activation is not the direct effect on the enzyme of adrenoblocking agents, but rather of the regulatory factor formed under their effect (Tables 1 and 2). The factor released from the vas deferens and atria activates ATPase in both the organs and the Na,K-ATPase of the rat brain, i.e. it has no organ specificity.

The change in ATPase activity produced by the effect of the regulatory factor can be detected only by the potentiometric method. This could be attributed to the fact that accumulation of protons is mainly the result of hydrolysis of the exogenous ATP of the surface ATPase of the cell membrane, whereas the concentration of inorganic phosphate in the incubation medium is the sum of the phosphate formed in the course of the hydrolysis of exogenous ATP and of that released into the medium at the hydrolysis of endogenic ATP in the whole tissue.

The possibility of ATP hydrolysis by the surface cell structures was reported by a number of authors. Thus, there were reports of the hydrolysis of exogenous ATP by the outer surface membranes of nerve terminals [13] and synaptosomes isolated from the chick brain [14].

The regulatory factor provides a certain level of Na,K-ATPase activity thus regulating the rate of neuronal uptake of noradrenaline. The factor consists of two components, the ratio of which determines the enzyme activity (Fig. 3). The maximal enzyme activation occurs only within the narrow range of the components concentration ratios. It seems that a change in the rate of formation of one or both of the components could regulate the enzyme activity, and, accordingly, all the physiological and biochemical processes related to it.

The high molecular mass (between 25 and 100 kDa), established by ultrafiltration, and the isopoints of two fractions of the regulatory factor point to its protein nature. The regulatory factor seems to be a system of two enzymes regulating Na,K-ATPase activity and through this, the level of neuronal noradrenaline and tyrosine uptake [1, 2]. A change in the activity of Na,K-ATPase depends on the ratio of the factor's fractions (Fig. 3).

To judge from the dependence pattern of Na,K-ATPase activity on the ratio of the fractions, the most probable mechanism of action of the factor seems to be a change in the membrane viscosity. This in turn could lead to conformational changes of integral plasma membrane proteins neurotransmitter transporters and Na,K-ATPase [15].

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