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Possible regulation of the myometrial Na^+/K^+-ATPase activity by Ca^{2+} and cAMP-dependent protein kinase

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We have previously reported an inhibition of the rat myometrial Na^+/K^+-ATPase by micromolar Ca^{2+} concentrations which was abolished by SDS treatment of the microsomal preparation. Application of dimethyl sulphoxide (DMSO) prevented this effect of SDS. In this report, we present our investigation into the mechanism of the inhibitory effect of Ca^{2+} on the myometrial Na^+/K^+-ATPase. We observed that, in parallel with inhibition by Ca^{2+}, phosphorylation of a number of membrane components was abolished by SDS treatment of the microsome fraction. Exogenously added calmodulin had no effect. However, the catalytic subunit of cAMP-dependent protein kinase restored Ca^{2+} sensitivity of the Na^+/K^+-ATPase and phosphorylation of the other components. Furthermore, addition of the heat-stable protein kinase inhibitor reduced drastically the Ca^{2+} sensitivity of the Na^+/K^+-ATPase, as well as the phosphorylation of a number of proteins in the myometrial microsome fraction. It is concluded that the cAMP-dependent protein kinase may be involved in the modulation of Na^+/K^+-ATPase activity by Ca^{2+} in the myometrial plasma membrane.

Introduction

The Na^+/K^+-ATPase is the biochemical manifestation of the active sodium pump operated essentially in all animal cells [1]. The structure and function of the Na^+/K^+-ATPase has been elucidated in more detail recently [2–4]. Relatively little is known, however, about the processes that are involved in the modulation of the enzyme activity. One form of control resides in the cellular mechanism that regulates protein synthesis and degradation. A second form of control is short-term regulation of the Na^+/K^+-ATPase, including processes which make possible the promt adaptation of the enzyme activity to the actual rate of ion transport required.

Over the last few years, it has been established that the activity of several enzymes is modulated by second messengers. Data concerning the kidney, brain, liver, cardiac and gastric smooth muscle show an undoubted influence of cAMP on the activity of Na^+/K^+-ATPase [5–8]. Phosphatidylinositol metabolism exerts also an effect upon the enzyme activity in several tissues [9–11]. On the other hand, Ca^{2+}, at extremely low concentrations, inhibits the Na^+/K^+-ATPase activity of red-cell membrane preparations in the presence of factor(s) derived from the hemolysate fraction [12–14]. Micromolar concentrations of Ca^{2+} can decrease the ouabain sensitivity of Na^+/K^+-

Abbreviation: DMSO, dimethyl sulphoxide.

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ATPase in the plasmocytoma cell membrane when the previously removed membrane component(s) were added to the assay system [15]. A 'digitalis-like' endogenous inhibitor may also be involved in the regulation of Na^+/K^+-ATPase activity, and its inhibitory effect has been demonstrated in several tissues [16–18].

These results, although requiring further characterization, suggest that the operation of the sodium pump could be controlled through different routes, and therefore may have a complicated regulation. These various ways of control are reasonable considering the several physiological functions of the Na^+/K^+-ATPase.

In our previous work, it was found that Na^+/K^+-ATPase activity measured in the microsome fraction of rat uterus smooth muscle was inhibited by Ca^{2+} at very low concentrations [19]. This property was not connected directly to the enzyme itself, and some other membrane-bound component(s) removable by SDS were found to be responsible for it. DMSO seemed to prevent solubilization of these factor(s) from the membrane. In the present work, we intend to characterize the mechanism of Ca^{2+} inhibition. The results indicate that the myometrial microsome fraction has a significant amount of endogenous protein kinase activity and several substrate proteins which can be phosphorylated under different conditions. The Ca^{2+} sensitivity of the Na^+/K^+-ATPase seems to be connected with phosphorylation of certain membrane component(s).

Methods and Materials

Isolation of plasma membrane fraction. Microsome fraction was obtained from rat myometrium by differential centrifugation, as described previously [19]. For preparation of plasma membrane, the microsomal pellet was washed with 0.3 M KCl, and layered on a 30% saccharose solution containing 50 mM sodium pyrophosphate and 0.1 M Tris-HCl (pH 8.3), then centrifuged at 100 000 $\times g$ for 120 min. Plasma membrane fraction enriched in the interface was collected and diluted with the homogenization buffer, comprising 0.25 M saccharose/30 mM histidine-HCl (pH 7.2). After centrifugation at 100 000 $\times g$ for 60 min, the pellet was suspended in the same medium.

SDS treatment. 1 mg of microsome, or plasma membrane fraction was treated with 0.60 mg SDS, in 1 ml, at room temperature for 10 min, according to Jørgensen [20]. The solubilized materials were separated by centrifugation at 100 000 $\times g$ for 60 min.

DMSO + SDS treatment. Before the addition of SDS, 1 mg of microsome fraction was mixed with DMSO to achieve a final concentration of 40% (v/v) in 1 ml at 0 °C, and this was then kept room temperature for 10 min, and centrifuged as above.

Determination of Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activity was determined in terms of the ATP hydrolysis inhibited by 1 mM ouabain. Reaction mixtures contained 100 mM NaN_3, 20 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 50 mM Tris-HCl (pH 7.4) with or without 1 mM ouabain. For measuring the Ca^{2+} effect, the reaction mixture was complemented with a Ca^{2+}/EGTA buffer system. Besides the materials listed, the test medium contained 1 mM EGTA, and the free Ca^{2+} concentrations were adjusted by addition of appropriate amounts of 0.1 M $CaCl_2$. The final concentrations of free Ca^{2+} were tested by Ca^{2+}-ion-selective electrodes (Radelkis, Hungary), and, at the lowest concentrations, were confirmed using Fura2 fluorescent indicator using the method of Grynkiewicz et al. [21]. 50 μg of protein was incubated in 1 ml of the reaction medium at 37 °C for 30 min. The reaction was stopped by addition of 100 μl 60% trichloroacetic acid, and the inorganic phosphate liberated was measured according to Lohmann and Jendrassik [22].

SDS-polyacrylamide gel electrophoresis. 50–100 μg of protein was boiled in the sample buffer comprising 10% (v/v) glycerol/5% (v/v) mercaptoethanol/2% SDS/20 mM Tris-HCl (pH 6.5). Electrophoresis at 50 mA was carried out in 12% polyacrylamide slab gels using the method of Laemli [23] for 5 h at 4 °C. Gels were dyed with 0.1% Coomassie blue dissolved in 50% (v/v) methanol/8.5% (v/v) acetic acid for 8–10 h. Fixation and differentiation were performed with 50% (v/v) methanol/8.5% (v/v) acetic acid and later with 5% (v/v) methanol/12.5% (v/v) acetic acid. Slabs were dried between two cellophane layers at room temperature.

Phosphorylation assay. 50–100 μg protein was

incubated at 0 °C in the presence of 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4) with 0.1 mM [γ-^{32}P]ATP in 150 μl for 10 min. The reaction was stopped by the addition of the sample buffer used for SDS gel electrophoresis. ^{32}P incorporation in the preparations was visualized by autoradiography of the dried gel slabs using Kodak X-ray film.

The protein content was estimated according to the method of Lowry et al. [24].

Materials. DMSO was purchased from Fluka, EGTA was obtained from Serva. Bovine serum albumin the catalytic subunit of cAMP-dependent protein kinase and its heat-stable inhibitor, were from Sigma. Calmodulin and calmidazolium were from Boehringer-Mannheim. [γ-^{32}P]ATP was from the Isotope Institute of the Hungarian Academy of Sciences. The chemicals not listed were from Reanal (Hungary) and were of analytical purity.

Results

Ca^{2+} sensitivity of myometrial Na^+/K^+-ATPase

In a previous report, we described conditions that made measurement of the myometrial Na^+/K^+-ATPase activity possible [19]. Briefly, we found that, if measured in the presence of 100 mM NaN_3, the background Mg^{2+}-ATPase activity was reduced greatly without any effect on the Na^+/K^+-ATPase, or on its Ca^{2+} sensitivity.

Fig. 1 shows the effect of Ca^{2+} on the Na^+/K^+-ATPase activity under different conditions. The Na^+/K^+-ATPase activity of the SDS-treated whole microsome fraction could be inhibited by 50% at a concentration of 2–5 μM Ca^{2+}. When the solubilized components were removed by centrifugation following detergent treatment, the membranous pellet maintained all the Na^+/K^+-ATPase activity, but the sensitivity to Ca^{2+} in the micromolar concentration range was almost completely lost. When SDS treatment was carried out in the presence of DMSO, the Ca^{2+} sensitivity of this pellet was unaffected.

The Ca^{2+} sensitivity of the Na^+/K^+-ATPase in the purified plasma membrane fraction followed essentially the same pattern as that of the microsome fraction (Table I), indicating that the phenomenon is associated with the plasma membrane.

From the above experiments, we conclude that some membrane component(s) are required for the Ca^{2+} sensitivity of the Na^+/K^+-ATPase.

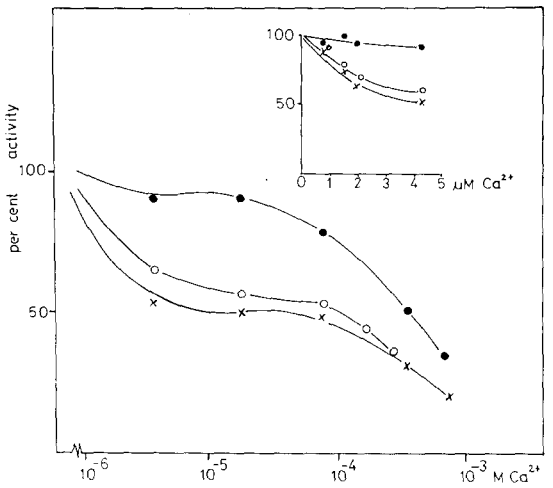

Fig. 1. Effect of Ca^{2+} on the Na^+/K^+-ATPase activity of the microsome fraction of rat myometrium under different conditions. The microsome fractions were treated with SDS or DMSO + SDS (see Materials and Methods). (○), SDS-treated whole microsomes; (●), pellet separated by centrifugation from SDS-treated microsome fraction; (×) pellet separated by centrifugation from DMSO + SDS-treated microsome fraction. Specific activities of the preparations were 6.2, 12.6 and 17.9 μmol P_i/h per mg, respectively. The effect of Ca^{2+} at micromolar concentrations can be seen in the inset.

We have tested whether the Ca^{2+} sensitivity of the Na^+/K^+-ATPase is related to calmodulin-dependent processes. As shown in Table II, exogenous calmodulin added to SDS-treated micro-

TABLE I

Na^+/K^+-ATPase ACTIVITY OF MYOMETRIAL MICROSOME AND PLASMA MEMBRANE FRACTIONS IN THE PRESENCE OF SDS: EFFECT OF Ca^{2+} ON THE ENZYME ACTIVITY

1 mg of microsome or plasma membrane fractions was preincubated in the presence of 0.6 mg SDS, in 1 ml, as described in Materials and Methods. 50 μl aliquots of this mixture were used for the enzymatic assay. For the conditions of ATPase assay, see Materials and Methods.

Preparation	Na^+/K^+-ATPase activity (μmol P_i/h per mg)		Inhibition by Ca^{2+} (%)
	1 mM EGTA	3 μM free Ca^{2+}	
Microsome fraction	6.7	3.7	45
Plasma membrane fraction	15.1	9.2	39

TABLE II

THE EFFECT OF CALMODULIN AND CALMIDAZOLIUM ON THE Ca^{2+} SENSITIVITY OF THE MICROSOMAL Na^+/K^+-ATPase ACTIVITY

For the preparation of DMSO + SDS, as well that of SDS-treated microsomes, see Materials and Methods.

Preparation	Addition	Na^+/K^+-ATPase activity (μmol P_i/h per mg)		Inhibition by Ca^{2+} (%)
		1 mM EGTA	3 μM free Ca^{2+}	
DMSO + SDS-treated ('Ca^{2+} sensitive')	none	18.9	9.6	49
DMSO + SDS-treated ('Ca^{2+} sensitive')	$5 \cdot 10^{-6}$ M calmidazolium	17.8	9.8	45
SDS-treated ('Ca^{2+} insensitive')	none	13.8	12.0	13.0
SDS-treated ('Ca^{2+} insensitive')	5 μg calmodulin	12.2	11.8	3.4

somes did not effect the Na^+/K^+-ATPase activity, or its insensitivity to Ca^{2+}. The calmodulin antagonist, calmidazolium, was tested on the DMSO + SDS-treated microsomes, and found not to affect the Ca^{2+} sensitivity.

In the light of reports on the modifying effect of cAMP-dependent phosphorylation on the Na^+/K^+-ATPase activity in a number of tissues, it seemed reasonable to test the effect of cAMP-dependent protein kinase on the Na^+/K^+-ATPase of myometrial microsomes [6,9]. As shown in Table III, the cAMP-dependent protein kinase catalytic subunit and its heat-stable inhibitor had no effect on the Na^+/K^+-ATPase activity of the SDS-treated preparation, which was separated further by centrifugation (i.e., 'Ca^{2+}-insensitive' preparation) in the absence of Ca^{2+}. However, Ca^{2+} sensitivity was restored by the cAMP-dependent protein kinase catalytic subunit. The heat-stable inhibitor prevented the restoration of Ca^{2+} sensitivity by the catalytic subunit.

Fig. 2 shows the effect of Ca^{2+} on the Na^+/K^+-ATPase activity of the SDS-treated ('Ca^{2+}-insensitive') preparation with and without the cAMP-dependent protein kinase catalytic subunit. In the presence of the protein kinase, 4.2 μM Ca^{2+} caused about 60% inhibition of the Na^+/K^+-ATPase.

Fig. 3 shows the effect of the protein kinase inhibitor on the DMSO + SDS-treated (i.e., 'Ca^{2+}-sensitive') preparation. It can be seen that the Ca^{2+} sensitivity of this preparation is abolished by the heat-stable protein kinase inhibitor.

These above results suggest that the Ca^{2+} sensitivity of the myometrial Na^+/K^+-ATPase is dependent, somehow, on the presence of a function cAMP-dependent protein kinase. Therefore, our attention was directed to examination of membrane components that could be phosphorylated by cAMP-dependent protein kinase and that

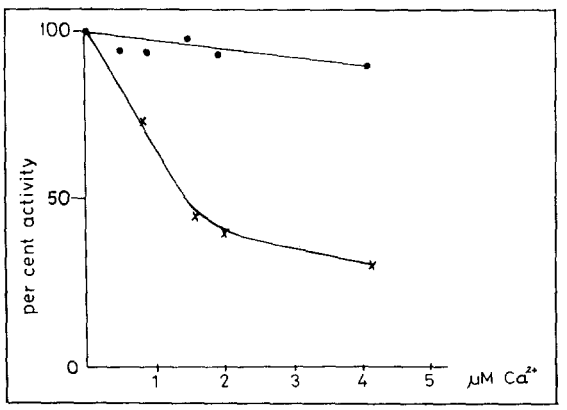

Fig. 2. The combined effect of Ca^{2+}- and cAMP-dependent protein kinase on the Na^+/K^+-ATPase activity of the 'Ca^{2+}-insensitive' preparation. Na^+/K^+-ATPase activity: (●), without and (×), with exogenous catalytic subunit of cAMP-dependent protein kinase. 100% activities were 17.0 μmol P_i/h per mg, in both cases. Catalytic subunit of cAMP-dependent protein kinase was added to the reaction mixtures, as described in the legend of Table III.

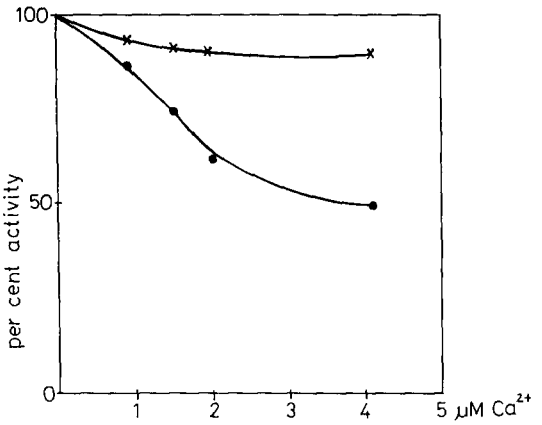

Fig. 3. Effect of cAMP-dependent protein kinase inhibitor on the Ca^{2+} sensitivity of Na^+/K^+-ATPase activity of the DMSO + SDS-treated ('Ca^{2+}-sensitive') preparation. Na^+/K^+-ATPase activity: (●), without and (×), with added protein kinase inhibitor. 100% activity without protein kinase inhibitor was 23.16, and with protein kinase inhibitor, 21.8 μmol P_i/h per mg. cAMP-dependent protein kinase inhibitor was added according to the legend of Table III.

TABLE III

THE EFFECT OF THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE AND ITS INHIBITOR ON THE Ca^{2+} SENSITIVITY OF Na^+/K^+-ATPase

Freshly dissolved 5 μg of the cAMP-dependent protein kinase catalytic subunit and/or 50 μg of its inhibitor in a solution containing 5 mg/ml dithiothreitol was added to the assay mixtures without K^+ and incubated with 50 μg of SDS-treated ('Ca^{2+} insensitive') preparation at 0 °C for 10 min. The control tubes contained dithiothreitol at appropriate concentration. The Na^+/K^+-ATPase assay was started by the addition of 20 mM KCl.

Addition	Na^+/K^+-ATPase activity (μmol P_i/h per mg)		Inhibition by Ca^{2+} (%)
	1 mM EGTA	5.3 μM free Ca^{2+}	
none	19.2	17.4	8.9
cAMP-dependent protein kinase catalytic subunit	18.8	5.8	69.2
cAMP-dependent protein kinase inhibitor	20.3	18.4	9.5
cAMP-dependent protein kinase catalytic subunit + inhibitor	19.6	16.9	13.8

might be involved in the regulation of the myometrial Na^+/K^+-ATPase.

Phosphorylation of microsomal proteins

Phosphorylation of 'Ca^{2+}-sensitive' and 'insensitive' preparations from rat myometrial, microsomes was analyzed by autoradiography following SDS gel electrophoresis.

As is illustrated in Fig. 4a, the protein composition of the 'Ca^{2+}-insensitive' and 'Ca^{2+}-sensitive' preparations was significantly different. Many proteins that were solubilized by detergent from

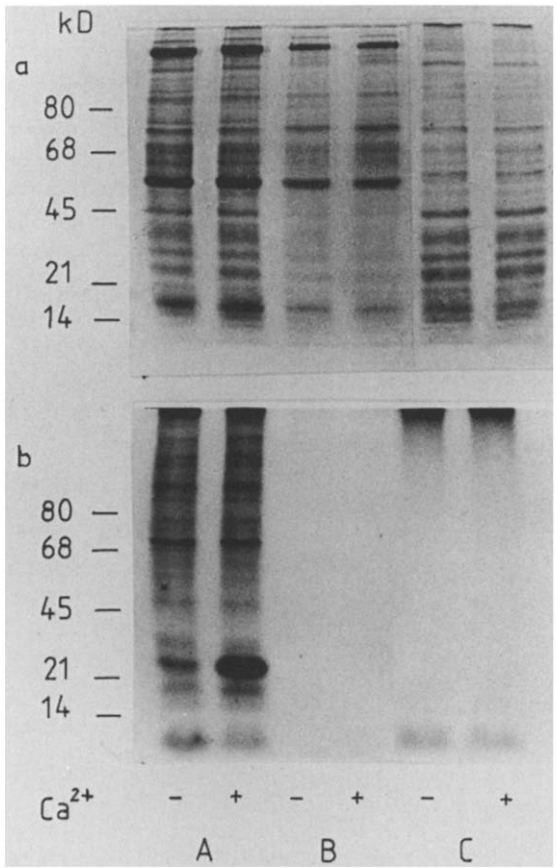

Fig. 4. Protein composition (a) and endogenous phosphorylation (b) of myometrial microsomal preparation. (A) DMSO + SDS-treated ('Ca^{2+}-sensitive') preparation; (B) 100000 × *g* supernatant after SDS-treatment of microsomes; (C) SDS-treated ('Ca^{2+}-insensitive') preparation. Phosphorylation and gel electrophoresis were carried out as described in Materials and Methods. The reaction mixtures contained 1 mM EGTA (−), or 5.3 μM free Ca^{2+} (+).

the microsomal fraction were found in the supernatant after centrifugation.

First, we examined the presence of endogenous protein kinase in the myometrial preparations. The microsomes were phosphorylated by [γ-^{32}P]ATP. As seen in Fig. 4b, high endogenous kinase activity was apparent in the DMSO + SDS-treated ('Ca^{2+}-sensitive') microsomes, while the incorporation of ^{32}P in the SDS-treated ('Ca^{2+}-insensitive') microsomes was very poor. In the presence of Ca^{2+}, phosphorylation of 20 kDa protein was greatly increased, indicating the presence of some Ca^{2+}-dependent kinase.

It was of interest to examine the effect of exogenously added cAMP-dependent protein kinase. In the SDS-treated ('Ca^{2+}-insensitive') microsomes, four proteins were phosphorylated by addition of the catalytic subunit of cAMP-dependent protein kinase. Two major substrates of the cAMP-dependent kinase could be observed: a 20 and a 68 kDa protein. Two other components (45 and 80 kDa) were phosphorylated also, but only weakly. These phosphorylated components were present in the DMSO + SDS-treated ('Ca^{2+}-sensitive') microsomes without addition of exogenous protein kinase (Fig. 5).

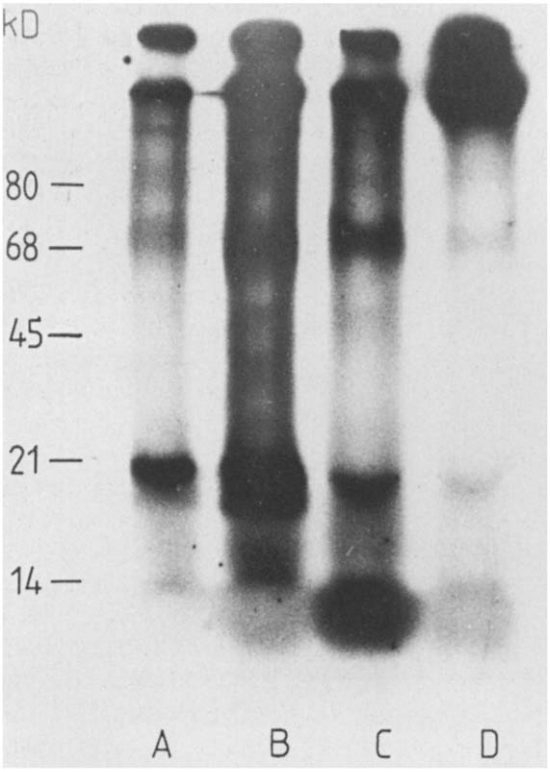

Fig. 5. Effect of catalytic subunit of cAMP-dependent protein kinase and its specific inhibitor on the phosphorylation of myometrial microsomal preparations. (A) DMSO + SDS-treated ('Ca^{2+}-sensitive') preparation plus cAMP-dependent protein kinase inhibitor (50 μg); (B) DMSO + SDS-treated preparation; (C) SDS-treated ('Ca^{2+}-insensitive') preparation plus cAMP-dependent protein kinase catalytic subunit (5 μg); (D) SDS-treated preparation. The samples were phosphorylated in the presence of 5.3 μM free Ca^{2+}, as described in Materials and Methods.

When the heat-stable protein kinase inhibitor was added to the DMSO + SDS-treated preparation, the high phosphorylation of the 20 kDa protein was scarcely influenced. Considering its molecular weight and its Ca^{2+}-dependent phosphorylation, it could be supposed that this protein was very similar to myosin light chain [25–26]. However, the intensities of the above-listed radioactive bands were significantly reduced in the presence of the inhibitor, indicating the cAMP dependency of their phosphorylation.

Discussion

Sensitivity of Na^+/K^+-ATPase to micromolar concentration of Ca^{2+} has been reported so far only in erythrocytes [12–14]. We have demonstrated that Na^+/K^+-ATPase measured in microsomal fraction of rat uterus smooth muscle could also be inhibited by Ca^{2+} in the micromolar range. The effective concentrations of free Ca^{2+}, determined by Ca^{2+}-ion-selective electrodes, proved to be lower than calculated in our previous report [19]. The values at the lowest concentrations of free Ca^{2+} were confirmed using Fura2 fluorescent Ca^{2+} indicator.

The results showed that, at most, half of the total Na^+/K^+-ATPase activity could be inhibited by about 5 μM free Ca^{2+}. Complete inhibition was observed at more than a 100-fold the concentration of Ca^{2+}. One possible explanation of this phenomenon might be the difference in the microenvironment of Na^+/K^+-ATPase in the different domains of the plasma membrane. Another suggestion is the existence of two isoenzymes of Na^+/K^+-ATPase found in several tissues [27–29]. The α + form differs from the α in form in having

a higher affinity for cardiac glycosides and ATP, a greater number of sulphydryl groups, and a different susceptibility to proteolysis [27,30]. It can be supposed that only one of the isoenzymes is inhibited by micromolar concentrations of Ca^{2+} in the myometrium.

The influence of the cAMP-dependent phosphorylation on the activity of Na^+/K^+-ATPase has been investigated by several groups. Na^+/K^+-ATPase activity in the brain, kidney medulla and liver was decreased in the presence of cAMP or cAMP-dependent protein kinase [6,9]. Adenyl cyclase-activating agents, such as isoproterenol and forskolin also reduced the Na^+/K^+-ATPase activity in several tissues [31]. On the contrary, the Na^+/K^+-ATPase of kidney cortex and gastric smooth muscle was activated by cAMP-dependent protein kinase [31,32]. In the plasma membrane vesicles prepared from heart muscle, cAMP-dependent protein kinase was ineffective on the ATPase activity, although certain membrane components were phosphorylated in parallel [33–34]. Other authors suggested that phosphorylation could enhance the Ca^{2+}-binding ability of heart Na^+/K^+-ATPase preparations [35].

Biochemical data concerning myometrial Na^+/K^+-ATPase are not available. Localization of adenyl cyclase in the myometrium has been established previously [36]. Its activity was influenced by catecholamines, depending on the actual hormonal state of uterus. Furthermore, cAMP-dependent protein kinase activity has been also detected [37].

Our data showed that cAMP-dependent protein kinase did not influence the Na^+/K^+-ATPase activity in Ca^{2+}-free medium in the myometrium, which is similar to the result reported in the case of heart muscle [34]. The inhibitory effect on myometrial Na^+/K^+-ATPase could be manifested only when both Ca^{2+}- and cAMP-dependent protein kinase were present. The sensitivity of Na^+/K^+-ATPase to micromolar Ca^{2+} was abolished almost completely following SDS treatment of the microsome fraction, with a concomitant disappearance of phosphorylation of membrane proteins. However, exogenously added catalytic subunit of cAMP-dependent protein kinase restored the Ca^{2+} sensitivity and enhanced the phosphorylation of certain proteins in the SDS-treated preparation. The proteins phosphorylated by both endogenous and exogenous cAMP-dependent protein kinase are supposed to be involved in the mechanism of inhibition at low concentrations of Ca^{2+}. Recently, many Ca^{2+}-binding proteins were demonstrated in the myometrial plasma membrane [38]. According to our opinion, some substrate(s) of the cAMP-dependent protein kinase not removed from the membrane by SDS may bear phosphorylation-dependent Ca^{2+}-binding properties, thereby regulating Ca^{2+} sensitivity of the Na^+/K^+-ATPase. The following steps of our investigation will be to characterize these substrates and to examine their Ca^{2+}-binding abilities, as well as to clarify the topical relations in the membrane among the adequate substrate(s), the protein kinase and the Na^+/K^+-ATPase.

Ca^{2+} sensitivity of myometrial Na^+/K^+-ATPase was independent of calmodulin. However, protein kinase C cannot be excluded from this effect, since it often phosphorylates the same acceptor proteins as the cAMP-dependent enzyme [39]. To clarify this point, appropriate investigations are in progress.

Physiological data, including ion-flux and muscle-contraction measurements, lead to the conclusion that the Na^+/K^+-ATPase takes part in the regulation of smooth muscle contraction. Inhibition of the Na^+/K^+-ATPase by ouabain, or by lowering the external K^+ level led to a rise in cell Ca^{2+}, and increased vascular resistance in dog arteries [40]. Catecholamines known as Ca^{2+}-mobilizing agents amplified this effect of ouabain [41]. In view of the physiological data, the extreme high Ca^{2+} sensitivity of the Na^+/K^+-ATPase reported here could play a considerable role in controlling intracellular Ca^{2+} levels in the myometrium by a possible regulation of the rate of Ca^{2+} efflux via an Na^+–Ca^{2+} exchange system. Furthermore the rise in the intracellular Na^+ levels by the partially inhibited Na^+ pump may be one of the events which contributes to the contraction of uterus smooth muscle.

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