3. O. N. Moskovets, "Electrophysiological analysis of the antinociceptive effects of electrical stimulation of the concha auriculae in cats," Author's abstract of dissertation for the degree of Candidate of Medical Sciences, Moscow (1981).
4. M. S. Buchsbaum, G. C. Davis, and W. F. Bunney, Nature, 270, 620 (1977).
5. H. Dehen, J. C. Willer, and J. Cumbier, Adv. Pain Res. Ther., 3, 553 (1979).
6. J. S. Han, G. X. Xie, X. Z. Dung, and S. Tan, Pain, Suppl. 2 (1984), pp. 369-371.
7. C. Takeshigi, M. Murai, Chang Pin Zuo, and H. Shimizu, Neurosci. Lett., 13, Suppl. 2, 431 (1979).
8. A. L. Vaccarino, R. A. Tasker, and R. Melzack, Pain, 36, 103 (1989).
9. X. Wang, Z. Zhou, and J. Han, Acupuncture Res., 14, 142 (1989).

CHARACTERISTICS OF SARCOLEMMAL ATPASE ACTIVITY OF LONGITUDINAL AND CIRCULAR MUSCULATURE OF THE CANINE ILEUM

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The enzyme Na,K-ATPase, responsible for energy-dependent electrogenic transport of Na^+ and K^+ across the plasma membrane, plays a fundamental role in the function of smooth-muscle cells [5, 10]. The catalytic properties of this enzyme from muscles of the uterus [8], blood vessesl [9], and stomach [12] have been investigated. Previously the present writers partially characterized the Na,K-ATPase activity of membrane preparations of the muscles of the canine ileum and discovered it to be specifically inhibited by acetylcholine (ACh) [1, 2]. On the whole, however, the Na,K-ATPase of smooth muscles remain incompletely studied because of the difficulty of isolating purified preparations of sarcolemma from this tissue, and the many stages involved in the process, and also because of the low activity of this enzyme in the preparations obtained, which is masked by the much higher Mg-ATPase activity [4, 12].

The aim of this investigation was to compare some properties of the ATPase activity of the sarcolemma of longitudinal and circular muscles of the canine small intestine, which have different mechanical and electrical characteristics [11, 15]. We also studied the effect of neurotransmitters ACh and serotonin (5-HT), which contract the smooth muscles of the ileum [7]. The experimental approach used in the work, consisting of a combination of a rapid method of isolating the sarcolemma and treating the membranes with sodium dodecylsulfate (SDS), enabled many of the difficulties associated with the study of smooth muscle Na,K-ATPase to be eliminated.

EXPERIMENTAL METHOD

Experiments were carried out on mongrel dogs weighing 5-8 kg, anesthetized with thiopental sodium (30 mg/kg). A segment of ileum (40-60 cm) was excised, the mucous membrane removed from it, and the circular and longitudinal muscles were then carefully isolated. This and subsequent procedures were carried out at 2-4°C. Preparations of the sarcolemma were isolated by the method in [13] in the following modification: 5 mM Tris-HCl, pH 7.4; 50 mM Na pyrophosphate; 1 mM dithiothreitol; 5 mM phenylmethylsulfonyl fluoride; 0.1 mM EDTA, 0.02% NaN_3. The tissue was homogenized in a homogenizer of "Polytron" type 5 times, for 5 sec each time, separated by intervals of 1 min. The homogenate was centrifuged in a stepwise sucrose density gradient (analytical version: 15, 30, 35, and 41%, practical version: 30 and 35%) in a bucket-rotor (VAC-602, East Germany) at 95,000g for 90 min. Membrane fractions floating between the homogenate and the 15% sucrose solution (F_1), 15% and

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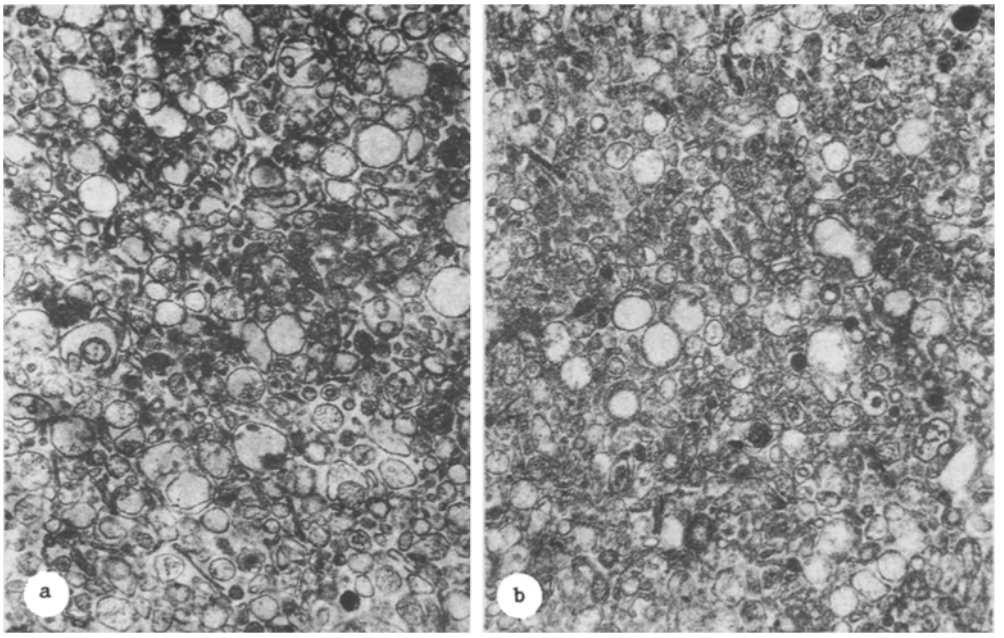

Fig. 1. Electron photomicrograph of preparations of sarcolemma (fraction F_3) of longitudinal (a) and circular (b) muscles of canine ileum. 24,000×.

30% (F_2), 30% and 35% (F_3), and 35% and 41% (F_4) sucrose solutions, and the residue (F_5) were collected, sedimented at 125,000g for 60 min, and washed under the same conditions. The residues were suspended in 2-4 ml of medium containing: 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.02% NaN_3, and 5 mM phenylmethylsulfonyl fluoride. The membrane preparations were kept at the temperature of liquid nitrogen.

Protein was determined by the biuret method in the presence of 1% sodium deoxycholate solution.

Total ATPase activity of the membrane fractions was determined by measuring accumulation of inorganic phosphate (P_i) by the method in [14] in 1 ml medium of the following composition: 50 mM Tris-HCl, pH 7.4; 100 mM NaCl; 0.5 mM Na_2-EDTA; 5 mM NaN_3, at 37°C. The reaction was started by addition of the membrane preparation and stopped by the addition of 1 ml of cold 3M acetate buffer, pH 4.3. Mg-ATPase activity was determined in the same medium in the absence of KCl, which was replaced with the equimolar concentration of NaCl. Na,K-ATPase activity was calculated as the difference between activities of total and Mg-ATPases. The total catalytic activity of Na,K-ATPase was measured in membranes treated beforehand with SDS (0.3 mg/mg protein) [6]. Na,K-ATPase activity was linear for 15 min with a concentration of membrane protein of 5-10 μg.

Succinate dehydrogenase (SDH) activity was determined by the method in [3]. Calcium uptake by preparations of the sarcolemma was measured with the aid of a Ca^{2+}-selective electrode (of the 20-15 "Crytur" type, Czechoslovakia) in medium: 100 mM KCl; 3 mM $MgCl_2$; 1.5 mM ATP; 20 μM $CaCl_2$; 5 mM potassium oxalate; 20 mM HEPES, pH 7.0, at 37°C. The reaction was started by the addition of the membrane suspension (200 μg protein).

For the electron-microscopic investigation residues of the membrane fractions were fixed in 2.5% glutaraldehyde solution followed by postfixation with 1% OsO_4 solution in phosphate buffer. The material was dehydrated in ethanols of increasing concentration and embedded in Epon-812. Ultrathin sections were stained with uranyl acetate and lead citrate and studied in the ÉMV-100 electron microscope (USSR).

EXPERIMENTAL RESULTS

To isolate the sarcolemma of the intestinal muscles, we modified the method in [13], developed for obtaining the plasmalemma of cells of nonmuscular nature. Table 1 summarizes the results of determination of the protein yield and activity of some marker enzymes in membrane fractions of homogenates of the longitudinal and circular muscles. The ATPase activity of the fractions was determined before and after treatment with a low SDS concen-

TABLE 1. Protein Yield, and ATPase (in μmoles P_i/mg protein/h) and SDH Activity (in nmoles succinate/mg protein/min) of Membrane Fractions of Homogenates of Longitudinal and Circular Muscles of the Canine Ileum (M ± m, n = 16)

Muscle	Fraction	Protein yield, mg/g tissue	Na, K-ATPase		Mg-ATPase		
			control	SDS	SDS	SDS	SDH
Longitudinal	F_1	0,31±0,04	0,62±0,11	2,44±0,12	0,51±0,07	0	0
	F_2	0,59±0,10	3,23±0,20	12,07±0,26	25,43±2,68	1,85±0,69	37,4±0,5
	F_3	1,72±0,21	6,65±0,60	24,11±1,13	57,60±4,71	2,90±0,02	48,4±3,3
	F_4	1,16±0,22	5,24±0,29	11,62±0,85	39,90±3,77	2,96±0,08	147,1±2,7
	F_5	81,60±0,51	2,44±0,06	8,86±0,57	2,11±1,05	0	459,2±55,4
Circular	F_1	0,47±0,06	1,02±0,05	4,26±0,28	0,51±0,01	0	0
	F_2	0,76±0,15	7,83±0,36	22,82±1,03	12,33±0,45	4,62±0,28	27,4±0,6
	F_3	1,94±0,36	14,37±0,21	39,85±1,34	32,81±1,37	3,97±0,36	32,3±2,5
	F_4	1,27±0,74	13,66±0,45	30,24±0,87	37,43±3,76	2,61±0,07	91,8±5,5
	F_5	73,30±1,27	4,84±0,05	7,54±0,49	5,57±0,37	0	528,0±28,3

Legend. SDS – 0.2 mg/mg protein.

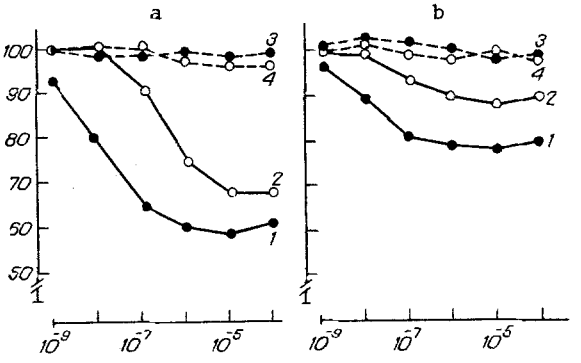

Fig. 2. Effects of ACh and 5-HT on Na,K-ATPase activity of sarcolemmal preparations (fraction F_3) of longitudinal (a) and circular (b) muscles of canine ileum. Each point is arithmetic mean of three parallel measurements, made in at least eight separate experiments. Values of standard errors do not exceed 10-12% of mean value. Abscissa, activity (in %); ordinate, concentration of agonists (in M). 1) Serotonin, 2) acetylcholine, 3) serotonin + methysergide (10^{-8} M), 4) acetylcholine + atropine (10^{-7} M).

tration with the aim of increasing membrane permeability [6], for in preparations of this kind the sarcolemma forms closed vesicles [6, 12], in which Na,K-ATPase centers, binding substrate and cations, may be inaccessible for these ligands. It will be clear from Table 1 that preincubation of the membranes with SDS led on average to a threefold increase of Na,K-ATPase activity. This indicates the predominantly vesicular organization of the sarcolemma in the resulting preparations, and this was confirmed also by the results of electron microscopy (Fig. 1).

In fractions F_2-F_4 the Mg-ATPase activity far exceeded the Na,K-ATPase activity (Table 1). This is in agreement with data in the literature [4, 12]. The detergent caused inactivation of Mg-ATPase, so that it was possible to determine Na,K-ATPase activity with greater accuracy.

Highest activity of Na,K-ATPase – a marker of plasma membranes – was observed in the F_3 fraction, in which activity of the marker of mitochondrial membranes (succinate dehydrogenase, SDH) was very low (Table 1). Evidence of the absence of any significant contamination of this fraction by mitochondria was given by the results of electron-microscopic analysis (see Fig. 1). Likewise no oxalate-stimulated Ca^{2+} uptake, characteristic of fragments of the sarcoplasmic reticulum (FSR; data not given), was found in F_3. Thus this fraction

was enriched to the greatest degree by sarcolemma and was considerably purified from mitochrondrial membranes and FSR.

Analysis of dependences of Na,K-ATPase activity of SDS-treated preparations of the sarcolemma (F_3) on the pH of the incubation medium and concentrations of Na^+, K^+, and Mg-ATP (data not given) showed that the optimum of this membrane activity is being determined under identical conditions in both longitudinal and circular muscles. In both cases, Na,K-ATPase activity was completely inhibited by the presence of 0.1 mM ouabain. Meanwhile its maximal value in the sarcolemma of the circular muscles was more than 1.5 times higher than in the longitudinal muscles (Table 1).

ACh and 5-HT induced a concentration-dependent fall of Na,K-ATPase activity in sarcolemmal preparations preincubated with SDS, which was more marked in the case of membranes from longitudinal muscles (Fig. 2). Sensitivity of the enzyme to 5-HT ($K_{0.5} \approx 1 \cdot 10^{-8}$ M) was an order of magnitude higher than to ACh ($K_{0.5} \approx 1.2 \cdot 10^{-7}$ M). The inhibitory effects of ACh and 5-HT were abolished by atropine ($1 \cdot 10^{-7}$ M), an antagonist of muscarinic acetylcholine receptors, and methylsergide ($1 \cdot 10^{-8}$ M), an antagonist of serotonin receptors respectively, evidence of functional coupling of these receptors with Na,K-ATPase in the sarcolemma of the smooth muscle. Mg-ATPase activity was not significantly changed by neurotransmitters.

Thus the relatively simple method used in this investigation to isolate plasma membranes yielded sufficiently well purified preparations of smooth muscles of the intestine, which, after treatment with SDS, have high Na,K-ATPase activity but virtually no Mg-ATPase activity. This approach can evidently be used also to study Na,K-ATPase from other types of smooth muscle.

The results of this investigation show that Na,K-ATPase activity of the sarcolemma of longitudinal and circular muscles of the canine ileum is characterized by identical kinetic parameters, but it differs essentially both in its maximal value and in its sensitivity to neurotransmitters. This is a factor which must evidently be taken into account when the mechanisms of regulation of movements of the small intestine are explained.

LITERATURE CITED

1. M. P. Danilenko, O. V. Esyrev, R. D. Omarova, et al., Byull. Éksp. Biol. Med., No. 9, 259 (1984).
2. M. P. Danilenko, Z. Sh. Samagulova, V. K. Turmukhambetova, et al., Ukr. Biokhim. Zh., No. 1, 55 (1988).
3. N. D. Eshchenko and G. G. Vol'skii, Methods of Biochemical Research [in Russian], Moscow (1982).
4. J. C. Allen and s. S. Navran, Trends Pharmacol. Sci., 5, 462 (1984).
5. C. Barajas-Lopez, E. Chow, A. Den Hertog, and J. D. Huizinga, J. Physiol. (London), 416, 369 (1989).
6. D. M. Bers, K. D. Philipson, and A. Y. Nishimoto, Biochim. Biophys. Acta, 601, 358 (1980).
7. T. B. Bolton, Phyisol. Rev., 59, 606 (1979).
8. M. E. Carsten and J. D. Miller, Arch. Biochem. Biophys., 204, 404 (1980).
9. R. C. Deth, J. L. Smort, C. J. Lynch, et al., Eur. J. Pharmacol., 99, 45 (1984).
10. W. W. Fleming, Annu. Rev. Pharmacol. Toxicol., 20, 129 (1980).
11. R. G. Gilbert, S. K. Sarna, and D. P. Harden, Am. J. Physiol., 252, G333 (1987).
12. C. Y. Kwan and P. Kostka, Mol. Physiol., 3, 265 (1983).
13. T. Maeda, K. Balakrishnan, and S. Mehdi, Biochim. Biophys. Acta, 731, 115 (1983).
14. W. B. Rathbun and M. V. Betlach, Anal. Biochem., 28, 436 (1969).
15. K. M. Sanders and T. E. Northrup, Am. J. Physiol., 244, G442 (1983).