Sodium pump isozymes are differentially expressed in electrically dissimilar regions of colonic circular smooth muscle

(colon/Na,K-ATPase/gene expression)

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ABSTRACT Molecular analyses of Na,K-ATPase abundance and α -subunit isoform distribution were performed to determine whether pump expression varies at different points through the thickness of the circular layer of colonic smooth muscle. The mRNA and polypeptides of Na,K-ATPase $\alpha 1$ and β subunits were twice as abundant in the submucosal region of the circular layer, which has previously been shown to generate large pump potentials. Sodium pump activity directly correlated with the relative abundance of the $\alpha 1$ polypeptide. These data show that sodium pump expression varies in electrically dissimilar regions of the circular layer.

Recent studies have suggested that the gradient in resting potential across the circular muscle layer of the canine colon is due to a gradient in the contribution of the Na,K-ATPase to the resting potential (1). Drugs or ionic conditions that inhibit the Na,K-ATPase abolished the resting-membrane potential gradient, such that all cells were polarized to approximately -40 mV (1). Several differences in cells through the circular layer might be responsible for the gradient in the contribution of the sodium pump to membrane potential. There may be differences in (i) pump expression in submucosal vs. myenteric muscle cells, (ii) membrane conductance in the two regions, (iii) concentration of pump substrates such as intracellular Na^+ , or (iv) Na^+ influx pathways such as differential expression of electrically silent transporters (e.g., Na^+/H^+ exchange).

The Na,K-ATPase couples the hydrolysis of ATP to the transport of three Na^+ ions out of the cell and two K^+ ions into the cell for each pump cycle (2, 3). This stoichiometry is the basis for its electrogenicity. Na,K-ATPase consists of a catalytically active α subunit ($M_r = 112,000$) and a glycosylated β subunit (protein component, $M_r = 35,000$). Several laboratories have identified cDNAs encoding three isoforms of the α subunit in rat brain [referred to as $\alpha 1$, $\alpha 2$ (or $\alpha +$), and $\alpha 3$] (4–7). Na,K-ATPase isozymes, which are composed of one α subunit ($\alpha 1$, $\alpha 2$, or $\alpha 3$) and one β subunit, have different affinities for sodium (8, 9). Thus distribution of the isozymes in the circular muscle layer should be determined, because differential expression of these isoforms of the pump may affect the magnitude of pump current and, therefore, the contribution of the sodium pump to resting potential. Experiments in the present study demonstrate the following: (i) at the RNA level, Na,K-ATPase $\alpha 1$ isoform and β subunit mRNAs are at least twice as abundant in submucosal circular muscle as in myenteric circular muscle whereas the $\alpha 2$ isoform is equally distributed throughout the circular muscle layer, (ii) at the protein level, Na,K-ATPase $\alpha 1$ protein is more abundant in submucosal circular muscle than in myenteric circular muscle whereas the $\alpha 2$ isoform is equally distributed, and (iii) there is approximately twice as much

sodium pump activity in submucosal circular muscle as in myenteric circular muscle.

MATERIALS AND METHODS

Tissue Preparation. Mongrel dogs of either sex were euthanized with sodium pentobarbital (45 mg/kg; i.v.). The abdomen was opened along the midline, and a segment of the proximal colon, 6–14 cm from the ileocecal sphincter, was removed. The segment of colon was opened along the mesenteric border and fecal material was removed with a Krebs–bicarbonate solution (KRB) containing 137.4 mM Na^+ , 5.9 mM K^+ , 2.5 mM Ca^{2+} , 1.2 mM Mg^{2+} , 134 mM Cl^- , 15.5 mM HCO_3^- , 1.2 mM H_2PO_4^- , and 11.5 mM dextrose. This solution had a pH of 7.3–7.4 at 37°C when bubbled to equilibrium with 97% O_2 /3% CO_2 . A rectangular piece of muscle was dissected from the sheet near the anti-mesenteric border, pinned-out in a dissecting dish, and bathed in ice-cold oxygenated KRB. Strips of muscle (≈ 1 mm $\times$ 40 mm) were cut parallel to the longitudinal fibers and turned on their sides to expose a cross section of the entire muscularis. The mucosal layers were removed, and the circular muscle was dissected into the following three strips: (i) a thin band of circular muscle adjacent to the submucosal border (referred to as “submucosal” strips), (ii) the central portion of the circular muscle (referred to as “bulk” strips), and (iii) the circular muscle adjacent to the longitudinal layer (referred to as “myenteric” strips). These dissections are described schematically in Fig. 1. The muscles were frozen in liquid nitrogen immediately after dissection.

Hybridization Analysis of α - and β -Subunit mRNA. Poly(A)⁺ RNA was prepared from dissected tissue by using the Fast Track kit (InVitrogen, San Diego) as described by the manufacturer's instructions. Muscles from four or five animals (0.5–1.0 g) were pooled for each RNA preparation. Each sample of RNA was adjusted to the same concentration. Qualitative Northern blot and quantitative slot-blot hybridizations were carried out as described (10). The α -isoform and β -subunit cDNA probes, provided to us by E. Benz (Yale University), were isolated from a rat brain library and have been characterized (6). Isoform-specific restriction endonuclease fragments were prepared from these probes according to Orlowski and Lingrel (11). The α - and β -subunit cDNA probes were labeled to similar specific activity with ^{32}P by using a random-primer technique (12). The filters were washed at high stringency [three 5-min periods in $2\times$ SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0)/0.1% SDS at room temperature and then two 30-min periods in $0.2\times$ SSC/0.1% SDS at 65°C] to assure specificity of labeling. A cDNA for CHO-B, a Chinese hamster ovary cell mRNA expressed at equivalent levels in all tissues examined (13), was used as an

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FIG. 1. Dissection of submucosal, bulk, and myenteric circular muscles.

internal standard to verify that equal amounts of poly(A)⁺ RNA were applied. Autoradiography was performed at -80°C with intensifying screens. Autoradiograms of slot blots were quantified by scanning densitometry. Significance was determined with Student's *t* test (unpaired) and accepted at a *P* < 0.05 level.

Western Blot Analysis. Crude microsomal membranes were prepared from the three types of muscle strips and used for Western blot analyses and Na,K-ATPase assays. Microsomal membranes were pelleted from crude homogenates prepared as described (10) at 100,000 × *g* for 1 hr and resuspended in the homogenization buffer for use in assays. Lowry assays (14) were performed to determine protein concentration. Equal amounts (40 μg per lane) of membrane protein were resolved by SDS/PAGE. The proteins were blotted onto nitrocellulose filters as described by Burnette (15) and probed with either a polyclonal rabbit anti-canine kidney holoenzyme antibody provided by R. Farley (University of Southern California) (16) or an α2-specific mouse anti-rat monoclonal antibody (McB2) provided by K. Sweadner (Harvard Medical School) and demonstrated to react with the canine α2 isoform (17). All blots were processed as described by Urayama (17) and quantified by laser densitometry. Quantitation of the α2 isoform by densitometry of immunoblots was unsuccessful due either to the low abundance of this isoform in the dissected muscles or the low affinity of the monoclonal antibody to the canine α2 isoform of Na,K-ATPase. However, since the monoclonal antibody against α2

demonstrated no nonspecific reactivity in immunoblots containing canine brain or heart microsomal protein, slot blots of protein applied directly to nitrocellulose filters were performed and incubated with the α2 antibody. These slot blots and the immunoblots were processed identically.

Enzyme Activity Measurements. Na,K-ATPase activity was assessed in a crude microsomal protein preparation by the measurement of K⁺-dependent *p*-nitrophenylphosphatase reaction of Murer *et al.* (18). This assay was chosen due to the large background nonspecific ATPase activity present in this tissue.

Graphical data are presented as mean ± SEM. Differences were determined by Student's *t* tests.

RESULTS

mRNA Abundance of Na,K-ATPase α1 and α2 Isoforms and β Subunit in Colonic Submucosal, Bulk, and Myenteric Circular Muscles. Northern blot analysis was performed to determine the specificity of the rat derived α-isoform and β-subunit cDNA probes to canine RNA and to assess the quality of the poly(A)⁺ RNA prepared from submucosal, bulk, and myenteric muscle strips. Fig. 2 displays autoradiograms of Northern blots probed with the indicated cDNAs. The molecular size of the α1 and β mRNAs is consistent with published values for canine Na,K-ATPase (10). However, whereas canine brain tissue displays the two transcripts characteristic of α2 expression, colonic smooth muscle ex-

FIG. 2. Autoradiograms of Northern blots containing mRNA from submucosal, bulk, and myenteric muscle strips and canine brain tissue probed with cDNAs for α1, α2, β, and CHO-B mRNAs as indicated. Lanes: S, submucosal muscle strips; B, bulk muscle strips; M, myenteric muscle strips; Brn, brain tissue. The positions of 18S and 28S rRNAs are indicated.

presses the 5.1-kilobase transcript predominately. A cDNA from CHO-B, a Chinese hamster ovary cell mRNA expressed at equivalent levels in all tissue examined (13), was used as an internal standard and is also shown in Fig. 2. Relative mRNA abundance was then quantitated for submucosal, bulk, and myenteric muscle strips by using the slot-blot technique. The data shown in Fig. 3 were compiled from three poly(A)⁺ RNA preparations. Each preparation was derived from tissue obtained from four or five animals. The results demonstrate that submucosal muscle strips express approximately twice as much Na,K-ATPase α 1-isoform mRNA as muscle strips dissected from the myenteric border. Na,K-ATPase α 2-isoform mRNA levels, however, were constant throughout the circular muscle layer. β -subunit mRNA expression levels demonstrate an even more pronounced difference between submucosal and myenteric strips than α 1 ($\approx$ 2.6:1). The nonstoichiometric differences in β -subunit expression relative to differences in α 1 has been described in other tissues (19) and may imply regulation at the level of Na,K-ATPase subunit assembly.

Polypeptide Abundance of the Na,K-ATPase α 1 and α 2 Isoforms and Na,K-ATPase Activity Levels in Colonic Submucosal, Bulk, and Myenteric Circular Muscles. Na,K-ATPase isoform distribution in submucosal, bulk, and myenteric muscles was determined by Western blot analysis. The stained α 1 immunoblot is shown in Fig. 4. The data shown in Fig. 5 were compiled from three microsomal protein preparations. Each preparation was derived from tissue obtained from four or five animals. The distribution of Na,K-ATPase α 1 polypeptide was similar to the distribution of α 1 mRNA. The amount of α 1 in submucosal muscle strips was significantly greater than that found in myenteric muscles. The α 2 polypeptide was quantitated from slot blots of microsomal protein applied directly to filters and incubated with monoclonal antibody against α 2 polypeptide. There is no statistically significant difference in α 2-isoform polypeptide abundance in submucosal, bulk, and myenteric muscles. Experiments were also performed to determine whether differences in RNA and protein expression are reflected in functional differences in sodium pump activity. Pump activity was assayed as units of K⁺-dependent *p*-nitrophenylphosphatase activity and normalized to the activity of submucosal muscle microsomes (Fig. 6). Pump activity correlated with the relative abundance of α 1 protein in submucosal, bulk, and myenteric muscles.

FIG. 3. Na,K-ATPase α -isoform and β -subunit mRNA levels in submucosal (Sub), bulk, and myenteric (Myen) muscle strips. An arbitrary value of 1.0 is given to submucosal mRNA abundance. mRNA abundance in bulk and myenteric muscle strips is normalized to that of submucosal muscle strips. *, Significance was determined with Student's *t* test (unpaired) and accepted at $P < 0.05$ level.

FIG. 4. Immunoblot analysis for α 1 isoform. Western blot contains 40 μ g of microsomal protein per lane. Lanes: S, submucosal muscle strips; B, bulk muscle strips; M, myenteric muscle strips; Kid, canine kidney. Position of the α 1 isoform is indicated.

In summary, we have determined Na,K-ATPase abundance at three levels of possible regulation: mRNA expression, polypeptide abundance, and enzymatic activity. In addition, we determined the relative levels of expression of the two Na,K-ATPase α isoforms present in this tissue. Although these data may not quantitatively reflect the levels of sodium pump activity *in vivo*, they do indicate heterogeneous expression of Na,K-ATPase genes in different regions of the circular smooth muscle layer.

DISCUSSION

Previous studies have demonstrated that circular muscles of the gastrointestinal tract are electrically and mechanically heterogeneous (20–24). In the present study we have described a possible molecular basis for part of the electrical heterogeneity: the gradient in resting potential that exists across the circular muscle layers of the proximal (21, 22) and distal (25) colon and stomach (26). The present study demonstrates that the Na,K-ATPase α 1 isoform and β subunit are differentially expressed at the RNA and protein levels in different regions of the circular layer. The α 1 isoform was most abundant in the region of muscle that displays the greatest contribution of the sodium pump to resting potential, and the expression of the α 1 isoform was significantly decreased in the region of muscle that displays little pump potential (1). These results provide evidence that the expression of some genes in circular smooth muscle cells is dependent upon their anatomical location and/or microenvironment. Regional differences in gene expression may provide an explanation for variations in electrical excitability (20–24), mechanical activity (20), and other cellular processes.

There may be a relationship between the gradient in resting membrane potential and the differences in sodium pump

FIG. 5. Na,K-ATPase α -isoform polypeptide levels in submucosal (Sub), bulk, and myenteric (Myen) muscle strips. An arbitrary value of 1.0 is given to submucosal polypeptide abundance. Polypeptide abundance in bulk and myenteric muscle strips is normalized to that of submucosal muscle strips. *, Significance was determined with Student's *t* test (unpaired) and accepted at $P < 0.05$ level.

FIG. 6. Na,K-ATPase-specific K⁺-dependent phosphatase activity in submucosal (Sub), bulk, and myenteric (Myen) muscle strips and canine heart and kidney microsomes. Data given are in terms of units of activity (μmol of P_i per mg per hr) and activity relative to the activity in submucosal muscle strips. Microsomal membranes prepared from myenteric muscle strips display Na,K-ATPase activity that is 57.8% that of microsomes prepared from submucosal muscle strips. *, Significance was determined with Student's *t* test (unpaired) and accepted at $P < 0.05$ level.

abundance in colonic muscles. Reports vary on the affinity of the Na,K-ATPase $\alpha 1$ and $\alpha 2$ isoforms for Na⁺ (27); however, Brodsky and Guidotti (9) reported that the $\alpha 2$ isoform of rat brain synaptosomes has a significantly lower affinity for Na⁺. The $\alpha 1$ form of the sodium pump had a $K_{0.5}$ for Na⁺ of 5–10 mM, and the $\alpha 2$ form had a $K_{0.5}$ for Na⁺ of 30–50 mM. Therefore, the $\alpha 2$ form of the enzyme is far less active at a physiological intracellular Na⁺ concentration. If the $\alpha 2$ isozyme displays similar kinetics in colonic smooth muscle, then depending upon intracellular Na⁺ levels in both regions, $\alpha 2$ may be considerably less active. Since there is a gradient in the expression of $\alpha 1$ and equal distribution of $\alpha 2$ through the circular muscle layer, smooth muscle cells at the myenteric border have a lower ratio of $\alpha 1/\alpha 2$ than cells at the submucosal border. Although the implications of this profile of pump expression on membrane potential also depend upon intracellular Na⁺ concentration and membrane conductance, it is possible that at least a portion of the gradient in pump potential found through the circular layer may result from the differential expression of pump isoforms.

Muscles of the proximal colon contain several cell types including nerve, interstitial cells (28, 29), and smooth muscle. The contribution of nervous tissue to the levels of Na,K-ATPase measured should be negligible because nervous tissue characteristically expresses abundant amounts of the Na,K-ATPase $\alpha 3$ isoform (27, 30), and the level of $\alpha 3$ isoform in all of the preparations studied was undetectable using a rat $\alpha 3$ cDNA probe that hybridizes to canine brain RNA. If one assumes little or no contamination from nervous tissues, then it is likely that smooth muscle is the predominant source of the RNA and protein measured because morphological studies have shown that smooth muscle cells are far more abundant than interstitial cells or any other cell type (28).

Our results suggest that factors that affect Na,K-ATPase gene expression may alter, perhaps pathologically, the electrical activity of colonic smooth muscle. For example, one pathological factor is thyroid status. Thyroid hormone is the major endocrine factor regulating Na,K-ATPase abundance. In the gastrointestinal tract, hyperthyroidism has been associated with hypermotility (31), which may be based on irregular electrical rhythm (32, 33). In many tissues, thyroid hormone increases Na,K-ATPase activity by stimulating an increase in the abundance of the enzyme (10, 34). It has been demonstrated that the Na,K-ATPase $\alpha 1$ and $\alpha 2$ isoforms are differentially regulated by thyroid hormone in a tissue-specific manner (35). It is possible that aberrant regulation of the Na,K-ATPase in circular muscles is responsible for the

pathological effects of hyperthyroidism in the gastrointestinal tract.

Another of the implications of differential gene expression in circular muscle is the possibility that ion-channel proteins are also differentially expressed. This could contribute, in part, to the heterogeneity in electrical activity in circular muscles throughout the gastrointestinal tract (21–24). Thus regulation of gene expression may be a critical factor in the physiology and pathophysiology of gastrointestinal motility.

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