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Inhibition of Ileal Brush-Border Chloride Conductance by Specific Antibody

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Summary. Antibody raised in mice was used in attempting to identify proteins responsible for the conductive chloride transport that can be measured in porcine ileal brush border membrane vesicles. Ileal brush-border membrane vesicle protein from pig was separated into five different molecular mass fractions by preparative SDS polyacrylamide disc gel electrophoresis. Separated protein fractions were used to immunize mice. Antibody was screened for reactivity with antigen by Western blotting, and for effects on conductive chloride transport in ileal brush border membrane vesicles. Immunization with brush-border protein from fraction I proteins (>110 kDa) produced polyclonal antisera which specifically inhibited the conductive component of chloride uptake by ileal brush border vesicle preparations. Western blotting of the antigen showed the presence of several protein species of molecular mass >100 kDa that were recognized by immune serum. Spleen cells from a mouse producing antiserum that inhibited conductive chloride transport were fused with a myeloma cell line. The resulting hybridoma colonies produced antibody that reacted with at least seven distinct protein bands by Western blot assay and inhibited chloride conductance in brush-border membrane vesicles.

Key Words exocrine chloride secretion · intestinal secretion · CFTR

Introduction

Specialized epithelial cells from a variety of exocrine tissues are responsible for the transport of electrolytes and water across mucosal membranes [2]. The rate of chloride ion efflux through an apical conductance channel may be the factor controlling the rate of primary fluid secretion by these exocrine tissues. Perturbation of the control systems for the chloride conductance channel is implicated in disease conditions such as cystic fibrosis and asiatic cholera [3, 14]. Characterization of this regulated ion transport protein would contribute to knowledge of basic disease processes of exocrine tissues.

Several proteins with reported chloride channel activity have been identified recently [4, 13, 15]. However no consensus exists as to which protein is primarily responsible for regulated exocrine chloride conductance. Precise identification of a specific regulated conductance protein in the enterocyte brush-border membrane is complicated by the large number of protein species in this tissue. We have previously demonstrated the presence of conductive chloride transport in porcine small intestinal brush-border membrane vesicles [5]. The brush-border membrane vesicle system lends itself to controlling conditions for chloride transport, and responds to inhibitors and physiological activators of this transport process [6, 7]. Ligand protection and differential labeling procedures have been used to indicate the involvement of a protein with a molecular mass of approximately 130 kDa in conductive chloride transport in porcine ileal brush-border vesicles [8]. The isolation of inhibitory antibody could provide definitive evidence for the involvement of a specific protein in conductive chloride transport.

Preparative electrophoresis was used to separate the total brush-border protein complement into smaller antigen groups. The resulting antisera have been screened for reaction with immunizing antigen and for inhibitory activity toward chloride conductance.

Materials and Methods

Preparation of Brush-Border Membrane Vesicles

Brush-border membrane vesicles were prepared as described previously [5]. Briefly, segments of porcine ileum were removed surgically from anesthetized weanling pigs of mixed Landrace-Yorkshire breeding. Mucosal scrapings were treated by a procedure of homogenization, differential centrifugation, and divalent cation precipitation. Apical membrane vesicles were used for antigen preparation, or equilibrated in appropriate buffers for transport studies.

Antigen Preparation

Brush-border membrane vesicle protein was separated according to molecular mass using preparative sodium dodecyl sulfate-polyacrylamide gel gel electrophoresis (SDS-PAGE). Membrane vesicle protein was electrophoresed on 7 × 10 cm 10% acrylamide gels [10]. After electrophoresis, gels were sliced into horizontal sections corresponding to the migration position of prestained standards. Slices (including fractions of the following molecular masses: I. >110 kDa, II. 84–110 kDa, III. 47–84 kDa, IV. 33–47 kDa, and V. 24–33 kDa) were obtained from eight separations. Corresponding slices were pooled, the protein was electroeluted from the polyacrylamide gel using 50 mM NH_4HCO_3 (pH 8.8) and 0.1% SDS, and concentrated to at least 2.0 mg per ml [12] by vacuum centrifugation. Antigen fractions I–V were stored at −20°C until used for immunizing mice.

Immunization and Antisera Collection

Seventy-five μg of antigen fraction emulsfied in Freund's complete adjuvant was injected intraperitoneally to female Balb/c mice. Secondary immunizations with 75 μg of antigen fraction emulsified in Freund's incomplete adjuvant were carried out at three-week intervals. Antisera were collected, and stored at −20°C until assayed by Western blotting [17] and examined for effects on conductive chloride uptake [5].

Western Blotting

Brush-border membrane vesicle protein (100 μg/lane) was separated by SDS-PAGE as described above, and transferred from the polyacrylamide gel to a nitrocellulose membrane by semi-dry electrophoresis. Nitrocellulose strips corresponding to protein lanes on the gel were cut and stored dry at 4°C until used for probing by antisera.

The nitrocellulose strips with bound protein were blocked with 3% bovine serum albumin, washed extensively with TBST (20 mM Tris pH 7.5, 0.5 M NaCl, 0.1% Tween 20), and incubated with primary antisera for 16 hr. The strips were subsequently washed again with TBST and incubated for 1 hr with rabbit anti-mouse IgG conjugated to alkaline phosphatase. The strips were washed and exposed to *p*-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Antisera Effects on Chloride Uptake

Brush-border membrane vesicles were prepared as described above. Membrane vesicles were equilibrated in medium containing either 100 mM K^+ or 100 mM tetramethylammonium (TMA). Valinomycin (7 μg/mg protein) was added to all vesicle suspensions 5 min prior to measuring Cl uptake. Ten or 20 μg of vesicle protein was used in each uptake measurement. Addition of uptake medium, consisting of 130 mM mannitol, 70 mM HEPES-tetramethylammonium (TMA) (pH 7.5), 100 mM K-gluconate and 10 mM TMA^{36}Cl (1 μCi/ml), produced a potassium gradent across the membrane of vesicles equilibrated in medium lacking K^+ [5]. The transmembrane potential generated by this K^+ gradient caused an increase in the initial rate of Cl uptake relative to initial uptake rates measured in vesicles with equal internal and external K^+ concentration. This increase in the initial rate of Cl uptake occurring in response to the transmembrane potential was defined as chloride conductance. Chloride conductance was reported as nmol of Cl trapped within the vesicles per mg of brush-border vesicle protein per 15 sec of exposure to conductance conditions. Antibody effects on Cl conductance were determined by mixing and precincubation prior to conductance measurements. Five μl of diluted antisera or hybridoma supernatant solution was mixed with 95 μl of brush-border vesicle suspension (200 μg of protein), and incubated with vesicle suspensions for 15 min at 20°C prior to measuring rates of Cl uptake. Uptakes were terminated after 10 sec by rapid filtration using 0.45 μm porosity mixed cellulose ester membrane filters, followed by three washes with 250 mM NaCl. ^{36}Cl was measured in a Beckman LS3800 β-counter.

Hybridoma Generation

One mouse, producing antisera that reacted positively in Western blot assays and inhibited chloride conductance, was given a final injection of 100 μg of antigen emulsified in Freund's incomplete adjuvant. Hybridomas were prepared using standard techniques [9]. Dispersed spleen cells from this mouse were fused with Balb/c cell line NS-1 cells by addition of 40% polyethylene glycol in RPMI 1640 medium (Sigma). The resulting hybridoma cells were grown in 96-well microtiter plates in complete medium including 100 μM hypoxanthine, 4.0 μM aminopterine, and 15 μM thymidine. Hybridoma supernatants were screened by a Western blot assay and also screened in a functional assay for effects on conductive chloride uptake into ileal brush border membrane vesicles.

Results

Brush-Border Protein Antigens

Porcine ileal brush-border protein was separated into five antigen fractions based on molecular mass. It was necessary to pool antigen fractions of the same size from several electrophoretic separations to obtain sufficient material for a complete immunization schedule. The protein composition of the pooled antigen fractions was investigated by SDS-PAGE and silver staining as shown in Fig 1. There were large numbers of protein species within each fraction, and there was some degree of overlap between fractions caused by the pooling procedure.

Polyclonal Antisera

Mice were immunized with each of the five antigen fractions shown in Fig 1. After a primary and three secondary immunizations, serum samples were collected and tested for antibody production by Western blotting. Total, unfractionated ileal brush-border protein was blotted to nitrocellulose. The specificity of polyclonal antisera for vesicle protein fractions

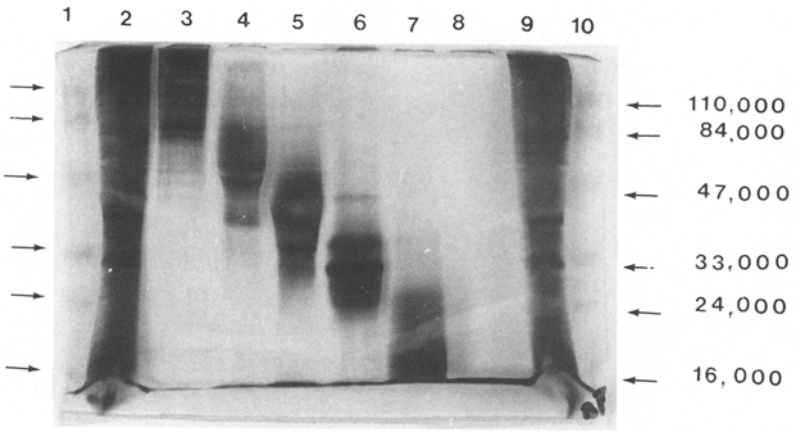

Fig. 1. Silver staining of electroeluted brush-border membrane vesicle proteins. Brush-border membrane vesicle protein was separated by SDS-PAGE, cut into regions based on molecular mass, electroeluted from the gel and analyzed for protein size distribution by SDS-PAGE and silver staining. Lanes *1* and *10:* molecular weight standards. Lanes *2* and *9:* 100 μg total vesicle protein. Lane *3:* 10 μg vesicle protein >110 kDa; lane *2,* 10 μg vesicle protein 84–110 kDa; lane *5,* 10 μg of vesicle protein 47–84 kDa; lane *6,* 10 μg protein 33–47 kDa; lane *7,* 10 μg protein 24–33 kDa.

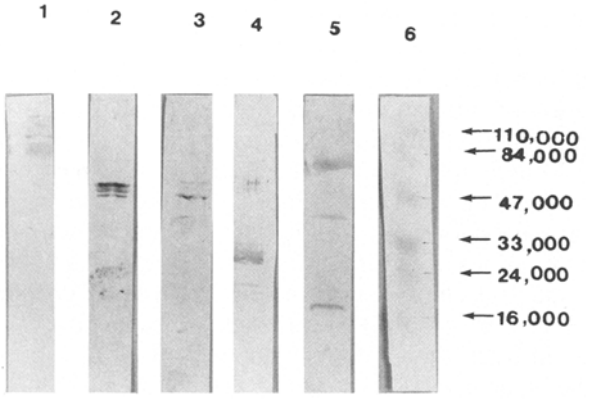

Fig. 2. Western blot of polyclonal antisera raised against size-fractionated brush-border vesicle protein. Total brush-border membrane vesicle protein was blotted to nitrocellulose and the blot screened with polyclonal antisera as described in Materials and Methods. All antisera were diluted to 1:3200 for blotting. Lane *1:* antisera to brush-border protein >110 kDa; lane *2,* antisera to brush-border protein 84–110 kDa; lane *3,* to brush-border protein 47–84 kDa; lane *4,* to brush-border protein 33–47 kDa; lane *5,* to brush-border protein 24–33 kDa.

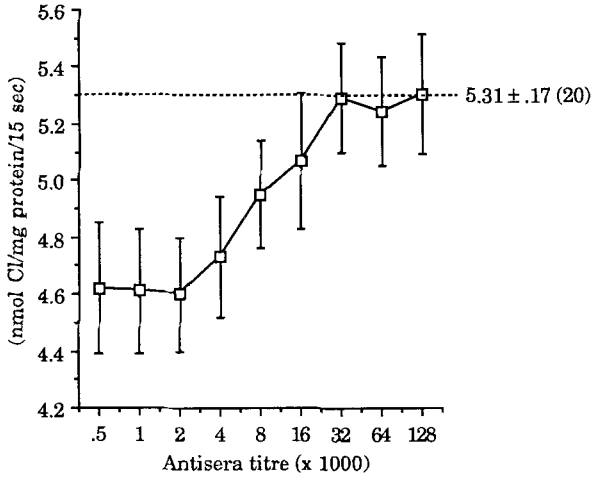

Fig. 3. Inhibition of the initial rate of total chloride uptake into ileal brush-border membrane vesicles by specific antisera. Vesicles were preincubated for 15 min at 20°C with increasing dilutions of antisera generated against ileal brush-border protein >110 kDa. After the preincubation and addition of valinomycin, initial rates of Cl^- uptake were measured in the presence of 100 mM extravesicular K^+ as described in Materials and Methods. Uptake rate in the control condition is shown by the dotted line. Points are means ± SE of 12 observations.

used in immunization is shown in Fig 2. Each of the antigen pools was immunogenic by this test criterion (Fig. 2). Specificity of the antisera was indicated by the positive reactions obtained between the antisera and the corresponding antigen fraction. There was also some apparent cross-reactivity between fractions. Antisera raised to fraction V cross-reacted with a small number of proteins in fraction I (lanes *1* and *5* in Fig. 2). Polyclonal antisera to the five fractions of vesicle protein were all detectable at a titre of 1/128000 in the Western blotting analysis; antisera to fraction I was detectable even at 1/256000.

Antisera Effect on Chloride Uptake

Pre- and post-immune antisera from the five fractions were tested for inhibitory properties against chloride uptake by brush-border membrane vesicles. All preimmune sera, and antisera fractions II to V had no inhibitory effect against chloride uptake occurring in response to the transmembrane potential generated by a 100 mM K^+ gradient. Antisera to fraction I, however, caused a significant decrease in the initial rate of chloride uptake in ileal brush-border membrane vesicles (Fig. 3). Uptake rates in the

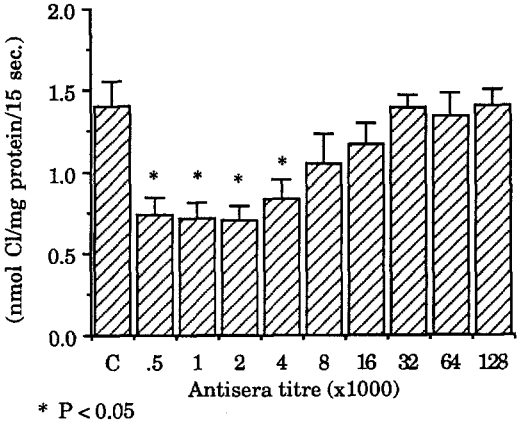

Fig. 4. The effect of specific antisera on chloride conductance activity. Vesicles and polyclonal antisera generated against brush-border protein >110 kDa were preincubated for 15 min prior to the addition of valinomycin, then ^{36}Cl. Chloride conductance was measured as the increase in the rate of chloride uptake driven by a K^+ gradient above the basal rate of chloride uptake occurring in the absence of a K^+ gradient ($n = 12 \pm$ SE of the differences). The rate of chloride conductance was reduced significantly in vesicles preincubated with antisera titres marked with an asterisk. ($P < 0.05$).

control condition are shown by the dotted line. The inhibition was detectable at an antiserum titre of 1/16000 and was maximal at a titre of 1/4000. Control rates of chloride uptake were observed in the presence of titres of 1/32000 or greater.

Nonconductive chloride uptake measured in pig ileal brush border vesicles in the absence of a K^+ gradient may represent activity of the chloride-bicarbonate anion exchanger or other anion carriers [5, 6]. This nonconductive component of chloride transport was not inhibited by any of the polyclonal antisera raised to antigen fractions I to V (*data not shown*). We have defined chloride conductance in this vesicle system as the difference between the rate of chloride uptake in the presence and the absence of a K^+ gradient [6]. This difference was chosen as a best approximation of conductive chloride transport in the system. Considering the lack of effect of immune antisera from fraction I (mass > 110 kDa) on nonconductive Cl^- uptake, together with inhibtion of the part of the chloride transport process that depends on a K^+ gradient, the result can be expressed as an antiserum effect on chloride conductance (Fig. 4). Antiserum to group I protein inhibited the conductive component of chloride uptake by as much as 50%, from 1.4 down to 0.7 nmol per mg protein per 15 sec at a titre of 1/2000. Exposure to fraction I immune sera did not affect equilibrated chloride space measured within the vesicles (*data not shown*).

Hybridoma Antibodies

The cell fusion experiment produced approximately 500 separate hybridoma colonies. There may be several different cell lines within each hybridoma colony prior to cloning, with each line producing distinct antibody. Supernatant solution from a selection of the colonies was tested by Western blotting to confirm the presence of antibody to the brush-border protein antigens. A selection of hybridoma colony supernatants reacted with at least seven unique protein species of molecular mass >110 kDa on nitrocellulose blots of unfractionated brush-border vesicle protein. Some representative banding patterns from antibody from hybridoma supernatant reacting with these protein blots are shown in Fig 5. At this stage, prior to cloning, most hybridoma supernatants reacted with more than one protein band.

Many of the hybridoma colonies that produced supernatant solution testing positive for antigen proteins of molecular mass >110 kDa were tested for inhibitory effects on chloride conductance. Preincubation of brush-border vesicle protein with hybridoma supernatant solution from colonies approaching 75% confluence in microtiter wells produced a range of effects on conductive chloride uptake by the vesicle suspensions. The control levels of conductance in this series of experiments was 3.11 ± 0.35 nmol of conductive chloride uptake per mg of vesicle protein per 15 sec ($n = 12$). Representative data from selected hybridoma colonies are presented in Fig. 6. The range of effects caused by preincubating hybridoma supernatant solution with brush-border vesicles varied from absent (well 5A2, bar *1*) to more than 90% inhibition of conductive chloride uptake (colony 2G6, bar *6*). Some strongly inhibitory colonies are being used in an ongoing study for limiting dilution cloning.

Discussion

The decision to sort antigen fractions prior to immunization was based on the complexity of the electrophoretic pattern obtained from separations of total brush-border vesicle protein. Preparative SDS-PAGE electrophoresis, followed by electroelution of selected areas, provided a simplified, molecular-size based grouping of brush-border proteins for use as antigens. Denaturing the protein with heat and SDS detergent prior to electrophoresis may have contributed to an increased immunogenicity of at least some of the proteins present in the fractions. High titres obtained with the antisera fractions in the Western blot assay indicated that it was possible to produce antibody with a reasonable affinity for at

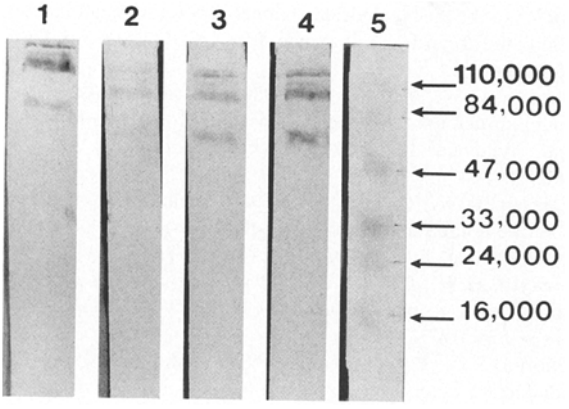

Fig. 5. Reaction of total brush-border vesicle protein with hybridoma supernatant. Brush-border membrane protein was separated by SDS-PAGE and blotted onto nitrocellulose. Hybridoma supernatant was collected from microtiter wells and diluted 1 : 4 for screening. Lanes *1–4:* four different well supernatants representing the full range of banding patterns. Lane *5:* prestained molecular weight standards blotted to nitrocellulose.

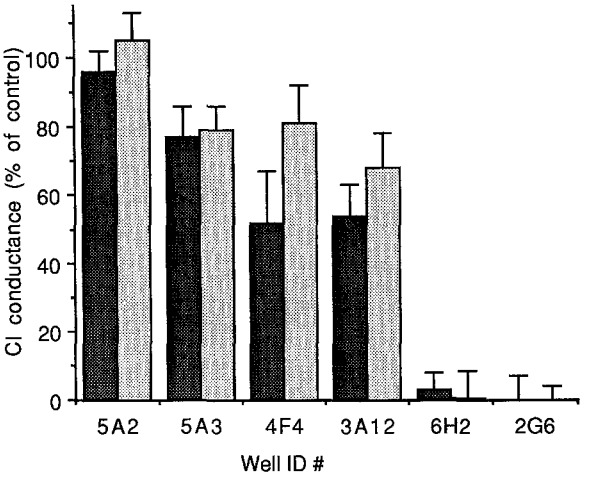

Fig. 6. Inhibition of conductive chloride uptake into ileal brush-border vesicles by supernatant solution from representative hybridoma colonies. Conductive chloride uptake was measured as described in Materials and Methods after a 15 min preincubation of hybridoma supernatant solution with ileal brush-border vesicle suspensions. Adjacent bars represent screenings of inhibitory activity of hybridoma supernatant from different passages of the same hybridoma colony. Values are means ± SE of the differences for four replicates of each measurement.

least some of the proteins present in the pig ileal brush-border preparation. Cross-reactivity detected between antibody raised to groups IV and V protein with group I antigen in Fig. 2 may have resulted from common glycoprotein epitopes on brush-border proteins of different size, or from limited proteolysis of the sample before electrophoresis.

Antibodies raised against a chloride conductance molecule denatured by heating in the presence of SDS could fail to recognize the native conformation of the molecule, or could bind to a part of the protein that would not affect a transport function. The finding of significant inhibition of conductive chloride uptake by one group of antisera indicated that important functional epitopes of the conductance protein were not irreversibly lost on preparative SDS-PAGE. The specificity of the immune sera effect was supported by a lack of inhibitory effects on chloride conductance by polyclonal antisera from groups II to V.

Several proteins have been associated with conductive chloride transport. Finn et al. [4] reported that monoclonal antibody raised to *Necturus* gall bladder epithelial cells inhibited chloride channels and reacted with proteins of molecular mass 219 and 69 kDa. The location of proteins identified by the monoclonal antibody on both apical and basolateral membranes by immunohistochemistry indicates a probable transport function for these antigens, but no further relationships to an apical conductance activity have been established. Landry et al. [11] used high affinity ligands for a chloride channels to identify and purify proteins which produced several distinct chloride channels on insertion into planar lipid bilayers. The resulting chloride channels did not show the expected sensitivity to chloride channel blockers that is characteristic of native chloride channels. More recently Ran and Benos [15] reported the isolation of a 38 kDa chloride channel protein from bovine tracheal membranes. The kinetics of ^{125}I transport in native bovine tracheal vesicles and in reconstituted liposomes was reported to be very slow (linear uptake for 10 min) in comparison to rates of conductive chloride transport in vesicles prepared from apical membrane of porcine small intestine (linear uptake for 15–20 sec) [5, 8]. A preliminary report of involvement of proteins of 58 and 62 kDa in chloride transport in the rabbit ileum has also appeared [13]. At the current stage in this area of research it is not clear whether there are several different chloride conductance proteins with quite different inhibitor sensitivities and conductance properties in different tissues, or if some of the techniques used to isolate and reconstitute putative ion channel proteins may be inducing channel activity in proteins that do not function in vivo as ion conductance channels.

The ability to produce complete inhibition of conductive chloride uptake by supernatant solutions of hybridoma colonies produced by fusion of spleen cells from an immunized mouse with myeloma cells

may be evidence for a limited number of chloride channels producing the conductive activity that is measurable in pig ileal brush-border vesicles. Variations in the degree of inhibition of the chloride conductance could arise from varying proportions of a strongly inhibitory cell line within the hybridoma cell population of one microtiter well. It is also possible that different degrees of inhibition represent recognition of more than one epitope of a chloride conductance protein, with corresponding differences in functional inhibition. We hope to resolve this issue by studying inhibitory activity of antibody produced by cloned hybridoma cell lines.

One goal of this line of investigation is the identification of a specific protein that is responsible for conductive chloride transport in exocrine tissues including the small intestine. Gross similarities in size of the pig ileal chloride conductance antigen and the cystic fibrosis transmembrane regulator (CFTR) anion channel [1, 16] make the CFTR one potentially interesting candidate as an antigen in our system. It may be possible to use monoclonal antibody with Western blotting and cDNA library screening to identify and determine the tissue and species distribution of the protein responsible for conductive chloride transport in the pig ileum.

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