Candidate Proteins for Conductive Chloride Transport in Porcine Ileal Brush-border Membrane*

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Conductive transport of chloride ion is important in controlling ion and fluid secretion by exocrine tissues. The current study was directed at identifying proteins in the intestinal brush-border membrane that may be involved with conductive chloride transport. Reaction of total brush-border membrane protein with phenylisothiocyaate inhibited conductive chloride transport into brush-border membrane vesicles. The conductive transport process was protected from this inhibition by including the ligands Cl⁻ and α-phenylacrylamide in the reaction mixture. Brush-border membrane protein protected by this procedure and labeled with fluorescein had an apparent molecular mass in the range of 130 and 23 kDa on separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Phosphorylation of brush-border membrane protein with [γ-³²P]ATP and endogenous protein kinase under conditions causing activation of chloride conductance in membrane vesicles caused the transfer of ³²P to several proteins, including ones in the same molecular size range (130 and 23 kDa) as those identified by the fluorescein labeling procedure. Conductive chloride transport in porcine intestinal brush-border vesicles may occur via proteins identified by this differential labeling procedure.

Electrogenic chloride ion secretion across the brush border of the small intestine has been studied extensively, and disturbances in chloride secretion have been implicated with abnormal fluid secretion occurring in clinical conditions ranging from cystic fibrosis to Asiatic cholera (1-4). However, little information is available about the transport protein responsible for electrogenic secretion of chloride ion or chloride conductance (5-7). Transport proteins present in the brush-border membrane have been identified by covalent labeling with a fluorescein reporter group (8,9). The approach involves the use of phenyl isothiocyanate (PITC), a nonfluorescent analog of fluorescein isothiocyanate (FITC) to prevent the reaction of FITC with nonspecific proteins. For this strategy to be successful, it is essential to have a molecule that can cause a reversible protection of the protein species chosen for labeling. α-Phenylacrylamide (α-PC) may be a suitable protectant molecule for a conductive chloride transport protein, because it has been shown to cause specific inhibition of conductive chloride uptake into brush-border membrane vesicles (10). In this study, α-PC was used to protect the chloride conductance transporter from reacting with PITC and to enhance the subsequent attachment of a fluorescein reporter group to brush-border membrane proteins that might be involved in chloride transport. The proteins labeled with fluorescein via reaction with FITC were separated by SDS-PAGE to identify candidates for a chloride conductance protein.

MATERIALS AND METHODS

Chemicals—Chemical buffers, FITC, and PITC were from Sigma. α-PC was from K & K Laboratories. H⁷Cl and [γ-³²P]ATP were from Du Pont-New England Nuclear. [¹⁴C]PITC was purchased from Amersham Corp.

Preparation and Chloride Uptake by Brush-border Membrane Vesicles—Brush-border membrane vesicles were prepared from mucosal scrapings of porcine jejunum or ileum by a procedure of homogenization, centrifugation, and divalent cation precipitation (11). Membrane vesicle purity was assessed by measuring the enrichment of marker enzymes as reported previously (12). Vesicles were resuspended in 150 mM mannitol, 70 mM HEPES-tetramethylammonium (TMA) (pH 7.5), and either 100 mM potassium gluconate or 100 mM TMA-glucinate to produce different ionic conditions for measurements of Cl⁻ transport. Valinomycin (1 μg/ml of protein) was added to vesicle suspensions 15 min prior to measuring Cl⁻ transport. Cl⁻ uptake was started by diluting vesicles with an equal volume of a solution of 130 mM mannitol, 70 mM HEPES-TMA (pH 7.5), 100 mM potassium gluconate, and 10 mM TMA⁺ (1 μM/ml).

Vesicles prepared in buffers described above with either 100 mM internal TMA or potassium ion were used to measure the increase in the rate of Cl⁻ uptake caused by an inside-positive membrane potential. Treatment of vesicle suspensions prepared in TMA with valinomycin and dilution in an equal volume of isotonic medium containing 100 mM K⁺ ion produced a membrane potential for driving conductive chloride uptake. This membrane potential was absent from vesicles already containing 100 mM internal K⁺ ion. Comparing uptake rates under these two conditions led to a definition of chloride conductance as the difference in the initial rate of chloride uptake measured in the presence and the absence of this imposed membrane potential.

Uptake of ³¹Cl⁻ was stopped by removal of the uptake medium from the vesicle suspensions via filtration on 0.45-μm porosity mixed cellulose ester membrane filters, followed by rapid washing with an isotonic NaCl solution. Filters were air-dried and the trapped radioactivity measured using a Beckman LS8000 β-counter.

Inhibitor Effects on Gradient and Nongradient Chloride Uptake—Varying concentrations of α-PC, ranging from 0.5 to 5.0 mM, were added to vesicles containing either 100 mM TMA-glucinate (gradient buffer) or 100 mM potassium gluconate (nongradient buffer) and allowed to equilibrate for 15 min. Initial chloride uptake rates were determined by adding vesicles to an equal volume of uptake medium.
and stopping the uptake after 10 s by dilution in excess isotonic NaCl solution and rapid filtration.

**Reaction of PITC with Brush-border Membrane Vesicles**—Brush-border membrane vesicles were diluted in 2.0 mM EDTA, 100 mM K₂HPO₄, and 50 mM Tris-HEPES (pH 9.2) (PITC buffer) to give a final protein concentration of 1.0 mg/ml. PITC was added from a stock [¹⁴C]PITC (2 μCi/ml) was used to quantitate PITC binding to brush-border membrane protein. Vesicles were collected on Whatman GF/C glass microfiber filters and washed repeatedly with isotonic NaCl. Filters were exposed with x-ray film for 28 h.

**PI TC/FITC Reaction with Brush-border Membrane Protein**—Brush-border membrane vesicles were diluted to a protein concentration of 1 mg/ml in PITC buffer as described above. Vesicles were treated with either no additions, 2.0 mM PITC, or 2.0 mM PITC with 5.0 mM α-PC and 5.0 mM KCl. After a 30 min incubation at 30°C, the solution was diluted 5-fold in PITC buffer and centrifuged for 30 min at 10,000 X g. The pellet was resuspended in 5 ml of PITC buffer and exposed to a subsequent reaction with 2.0 mM [¹⁴C]PITC (2 μCi/ml). For some experiments, 50 μM FITC was substituted for 2.0 mM PITC in this secondary labeling step to replace the [¹⁴C] label with a fluorescent label. The secondary reaction was allowed to continue for 1 h at 30°C followed by a 10-fold dilution in PITC buffer and centrifugation at 10,000 X g for 30 min. The pellet was assayed directly for [¹⁴C]PITC binding to protein by collection on glass microfiber filters, or the pellet was resuspended in gradient buffer, recentrifuged at 10,000 X g for 30 min, and used in chloride transport measurements. Brush-border membrane protein labeled with fluorescein was processed by resuspending the pellet in 30 ml of distilled water, centrifuging at 10,000 X g for 30 min, and denaturing with SDS and 2-mercaptoethanol prior to separation by gel electrophoresis.

**Densitometry of Brush-border Membrane Protein Labeled with Fluorescein**—SDS-polyacrylamide gel electrophoresis (PAGE) of membrane vesicle protein containing the fluorescein reporter group was carried out on 10% polyacrylamide gels with 100 μg of protein per lane. Gels were vacuum dried after electrophoresis and exposed to x-ray film. Bands were quantitated with a Shimadzu C930 scanning densitometer modified for autoradiography. The gel was run in each lane of SDS-PAGE. Gels were vacuum dried after electrophoresis and exposed with x-ray film for 28 h.

**RESULTS**

**Vesicle Properties**—The specific activity of the brush-border marker enzyme alkaline phosphatase was increased 14-fold in ileal brush-border vesicles prepared by this procedure. There was a 5-fold decrease in specific activity of cytochrome c oxidase and a 3-fold decrease in NADPH cytochrome c reductase specific activity in comparison with the homogenate of mucosal scrapings (12). Na,K-ATPase specific activity was increased 4-fold.

**Effect of a Potential Gradient on Chloride Uptake**—Chloride uptake by membrane vesicles was measured in the absence and the presence of an inside-positive membrane potential induced by a potassium gradient in the presence of valinomycin. Addition of vesicles prepared with internal 100 mM K⁺ to uptake media containing 100 mM K⁺ gave the nongradient condition, while the gradient was obtained in vesicles containing TMA as the internal cation. The initial velocity of chloride uptake increased significantly over the control rate when vesicles were exposed to this potential gradient (Fig. 1) (11). Under conductive conditions, the initial rate of Cl⁻ uptake appeared to be linear for at least 15 s. Measured uptake rates produced t₁/₂ values of 35 and 75 s, respectively, for the control condition and the K⁺ gradient condition. The stimulatory effect of the K⁺ gradient on the rate of Cl⁻ uptake was eliminated when valinomycin was omitted from the vesicle suspension (data not shown). Vesicles in the control condition with equilibrated internal and external K⁺ had a slower initial rate of chloride uptake but at equilibrium contained the same concentration of Cl⁻ as the vesicles exposed to the K⁺ gradient. Equilibrated chloride and glucose spaces of the vesicles were identical at 1.1 ± 0.2 gl/mg of vesicle protein (data not shown).

**Effect of α-Phenylcinnamate on Chloride Uptake**—The effect of a range of α-phenylcinnamate concentrations on the initial rate of chloride uptake occurring in the nongradient and in the gradient (inside-positive membrane potential) condition is shown in Fig. 2. Increasing concentrations of α-PC selectively decreased the initial rate of chloride uptake by vesicles with the inside-positive membrane potential. This effect occurred with concentrations of α-PC that were substantially lower than the chloride concentration in the uptake medium. α-PC up to 5.0 mM did not decrease the control rate of chloride uptake occurring in the absence of a potential gradient across the vesicle membrane and had no effect on the vesicle chloride content at equilibrium (data not shown).

The initial rate of chloride uptake was inhibited consistently by α-PC even with chloride concentrations as high as 50 mM. Previous analysis of this inhibition has indicated that it may be competitive with a calculated Kᵦₛ (dissociation constant for interaction of inhibitor with enzyme-substrate complex) of approximately 1.0 mM (10).

**PI TC Reaction with Brush-border Membrane Vesicle Protein**—The saturation of reactive sites for PITC attachment to protein was determined by varying the concentration and volume of PITC in the reaction system (Fig. 3). Accessible sites for reacting with PITC appeared to be maximal at a

![Figure 1](image-url)  
**FIG. 1.** The effect of a potential gradient on chloride uptake by ileal brush-border membrane vesicles. Chloride uptake was measured in vesicles equilibrated in either 100 mM potassium gluconate (○) (nongradient condition) or 100 mM TMA gluconate (●) (gradient condition). The inset shows the Cl⁻ content of vesicles with the inside-positive membrane potential (gradient condition) for the first 15 s of the uptake. Points are averages ± S.E. for 12 observations.
Ileal Brush-border Chloride Conductance Protein

**FIG. 2.** α-Phenylcinnamate effect on conductive and non-conductive chloride uptake. Vesicles were prepared as described and equilibrated in either gradient medium (○) or nongradient medium (□). α-Phenylcinnamate concentrations in the uptake media were obtained by adding a fixed volume from stock solutions to the vesicle suspension 15 min prior to the initiation of uptake. Points are averages ± S.E. for 12 observations.

**FIG. 3.** Effect of PITC concentration on the reaction with membrane protein. Vesicles were mixed with the indicated concentrations of [3H]PITC (2.0 pCi/ml). At the completion of the reaction, vesicles were washed, collected, and assayed for [14C]PITC content. Points are averages of five observations.

level of 600 nmol of PITC/mg of protein. Increasing the concentration of PITC above 2.0 mM did not substantially increase the bound PITC.

The reaction of PITC with brush-border vesicles caused a significant reduction in the initial rate of chloride conductance (Fig. 4). The initial rate of conductive uptake was inhibited by 25% (2.78 nmol of Cl⁻/mg of protein/10 s versus 3.62 nmol of Cl⁻/mg of protein/10 s) when 2.0 mM PITC was allowed to react with brush-border membrane vesicles. Addition of 5.0 mM α-PC and 5.0 mM KCl to the medium during the reaction with PITC prevented the inhibition of conductive chloride uptake. The initial conductance rate returned to control levels (3.52 nmol of Cl⁻/mg of protein/10 s versus 3.62 nmol of Cl⁻/mg of protein/10 s) after washing the vesicles to remove α-PC and unreacted PITC from the system.

Total labeling of vesicle protein by [14C]PITC produced a level of 710 nmol of PITC/mg of protein (Fig. 5). The secondary labeling with [14C]PITC was reduced by more than 75% to 165 nmol of PITC/mg of protein when brush-border membrane vesicles were reacted with 2.0 mM PITC in a primary treatment. The addition of α-PC and KCl to the primary treatment condition of 2.0 mM PITC increased the number of reactive NH₂ groups available for secondary labeling by [14C]PITC to 230 nmol of PITC/mg of protein. These data could be interpreted to indicate that the pre-treatment with α-PC and KCl protected brush-border membrane vesicle protein from primary binding by PITC to yield a 40% increase in secondary labeling. This increase in secondary labeling after protection corresponded to approximately 9% of the total reactive groups available for PITC conjugation in the vesicle preparation.

**FIG. 4.** Effect of reacting vesicle protein with PITC on the rate of conductive chloride uptake. Vesicle protein was allowed to react with 0 mM PITC (A), 2.0 mM PITC (B), or 2.0 mM PITC (C) and the ligands, 5.0 mM α-PC and 5.0 mM KCl. After the reaction vesicles were washed, divided, equilibrated in gradient and nongradient buffers, and assayed for initial rates of chloride uptake. Points are averages of the differences between initial chloride uptake rates measured in the presence and in the absence of a potential gradient. n = 6 replicate experiments ± S.E. (*, different from control; p < 0.05).

**FIG. 5.** PITC labeling of brush-border membrane vesicle protein. Vesicles were pretreated with 0 mM PITC (A), 2.0 mM PITC (B), or 2.0 mM PITC (C) and the ligands, 5.0 mM α-PC and 5.0 mM KCl. All pretreated vesicles were then exposed to a secondary labeling step with 2.0 mM [3H]PITC (2 pCi/ml). Vesicles were washed, collected, and assayed for [3H]PITC binding. Points are averages of six replicate experiments ± S.E.
to protein regions with molecular masses of approximately 130 and 23 kDa. The extent of the differential labeling in Fig. 6B of the protein in the high molecular weight range was enhanced significantly in vesicles competent for in vitro activation of chloride conductance (14).

Identification of Protein Kinase A Substrates in Brush-border Protein—Chloride conductance can be activated in vitro in vesicles prepared in I-A buffer, but ATP and cAMP addition do not increase conductance in vesicles prepared in HEPES-TMA (14). Activation of cAMP-dependent protein kinase under conditions that increased chloride conductance in vitro (14) showed several ileal and jejunal protein species to be substrates for protein kinase A (Fig. 7). The molecular mass regions identified by differential fluorescein labeling (130 and 23 kDa) are among the regions with a higher intensity of $^{32}$P in ileal brush-border protein from vesicles prepared in I-A buffer in comparison with the same amount of ileal protein from vesicles prepared in HEPES-TMA buffer. This apparent difference in $^{32}$P labeling intensity was not as clear in jejunal brush-border protein (Fig. 7, lanes 3 and 4).

**DISCUSSION**

Initial rates of ion uptake into a closed vesicle system are dependent on the number and the activity of transmembrane carriers for Cl⁻ ion, on the chemical and electrical gradients established across the vesicle membrane, and on the vesicle geometry. The Cl⁻ transport studies in this vesicle system were designed to give an estimate of the number and activity of chloride channels by determining the effect of a positive internal membrane potential on the initial rate of Cl⁻ uptake. Differences in initial rates of Cl⁻ uptake can be attributed to the effect of the membrane potential if external Cl⁻ concentration and vesicle geometry are held constant. However, the different buffer conditions required to generate the potential gradient across vesicle membranes could be affecting the size of vesicles. This concern was addressed by measuring the Cl⁻ content of vesicles at equilibrium conditions. Equal values for Cl⁻ content at equilibrium (chloride space) gave a simple confirmation that the buffer manipulations necessary to induce the membrane potential did not cause significant changes to vesicle size.

The conductive transport rates observed in these vesicles are much lower than would be expected for typical chloride channels measured by patch clamp procedures. A single 40-picosiemens chloride channel per vesicle would allow equilibration of chloride across the vesicle membrane in a few milliseconds. If such channels are responsible for the chloride transport observed in ileal membrane vesicles, then they must exist largely in a closed state and open for relatively short periods of time. It is important to note that there is a good agreement between conductivity values observed in vesicle systems and in intact mucosal sheets when the transport rates measured in the vesicle preparations are expressed per unit amount of vesicle surface area and compared with short circuit currents measured per unit area in sheets of ileal mucosa (14).

The differential labeling strategy used by Peerce and Wright (8, 9) to identify the Na-glucose cotransporter in brush-border membrane depended on the use of Na⁺ and glucose as specific ligands for protection of the cotransporter during the initial reaction with FITC. Chloride ion would not be expected to provide specific protection to a chloride conductance protein. Intestinal brush-border membrane may contain several proteins including the Cl⁻/HCO₃⁻ anion exchanger with potential binding sites for Cl⁻ (11). The differential labeling procedure would be feasible only if there were...
a specific ligand capable of protecting the chloride conductance channel.

There are some difficulties associated with measuring ligand specificity in the vesicle system. Porcine ileal brush-border membrane vesicles have a fairly large capacity for chloride transport in response to only the chemical gradient for Cl⁻. This component of the initial rates has been subtracted in our conductance measurements because the tissue is known to have chloride transport systems such as a Cl⁻/HCO₃⁻ antiport that should not depend on membrane potential for activity (11). We recognize that there could be limited Cl⁻ transfer occurring via a conductance unipur in the control state with no transmembrane potential, but this component of the initial uptake rate measured under control conditions has been neglected in order to focus on ligands that show specificity for the part of the rate that depends on the membrane potential. The definition of conductance in this study was the difference in the initial rate of chloride uptake measured in the presence and the absence of an imposed membrane potential.

Agents such as SITS, DPAC, and NPPB have been reported to be antagonistic ligands for conductive chloride uptake (15, 16). However, they lack specificity in the porcine ileal brush-border vesicle system (10) and would have limited usefulness as reversible protective agents for a chloride conductance process. The specificity of α-PC for the conductive portion of the Cl⁻ uptake supported the use of this compound as a protective ligand in the labeling experiments.

Kinetic analysis indicated that α-PC was an uncompetitive inhibitor of conductive chloride uptake (10). Uncompetitive inhibition is defined as binding of the inhibitor to the enzyme-substrate complex. The combination of α-PC and chloride could form an enzyme-inhibitor-substrate complex to act as a protective ligand against reaction of isothiocyanate probes with a conductive chloride channel.

After choosing a suitable ligand for the conductance protein, the next requirement in the differential labeling strategy was to find a suitable reactive reporter group that could alter Cl⁻ transport in the vesicle system. PITC met this requirement by inhibiting conductive chloride uptake after reacting with vesicle protein.

The third essential condition for differential labeling involves competition between the protective ligand and the reactive reporter group. The ability of the ligands to prevent the inhibition of chloride conductance activity caused by reaction with PITC implied that the presence of ligands induced steric restrictions on specific amino groups in the channel protein, causing reduced reactivity to PITC. Combined evidence from the effects of channel ligands to protect functional transport activity and to protect reactive NH₂ groups for secondary labeling could indicate that occupation of a ligand-binding or transport site on the conductance protein may selectively protect some amino groups from a primary reaction with the isothiocyanate derivative. The presence of reactive amino groups close to the Cl⁻-binding site of this transport process would not be unexpected. Positively charged groups would be required in the active site to interact with the chloride ion. Studies of inhibitory compounds have also suggested the role of positive charges at the active site of the Cl⁻ channel (10, 16).

Apparent competition between PITC and the conductance ligands α-PC and KCl was confirmed by the increase in secondary labeling occurring after a washout of the ligands. The degree of protection was relatively large, at 65 nmol/mg of vesicle protein, or about 9% of the total groups reacting with PITC. We have no reason to believe that the chloride conductance protein contributes 9% of the reactive NH₂ groups on the vesicle surface, and in fact the lack of previous identification probably implies a much lower abundance of this protein. This discrepancy between expected abundance of the protein and the amount of protected amino group raises some concerns about the use of differential labeling for identifying the chloride conductance protein.

Two specific regions with enhanced fluorescein labeling were identified when vesicles prepared in HEPES-TMA buffer were run in the secondary reaction with FITC. The absence of a differential fluorescein label on the 23-kDa protein from the imidazole-acetate vesicles raises questions about the relevance of this protein to conductive chloride transport. However, the larger of the two proteins identified by this procedure corresponded, within the resolution capacity of the system, to the protein size range showing differential fluorescein labeling in vesicles prepared under conditions permissive for in vitro activation of conductance. Enhanced amounts of fluorescein attached to this protein in vesicles permissive for in vitro activation could indicate a connection between conductive chloride transport and activation of chloride conductance. Precise identification of the major protein band(s) carrying the differential fluorescein label will require separation of membrane protein under conditions with greater resolving power.

As observed by other investigators (17–19) there was no specific specificity of labeling of brush-border protein with 32P by endogenous protein kinase. We have shown that it is possible to carry out in vitro activation of chloride conductance in vesicles prepared in imidazolium-acetate buffer. However, vesicles prepared in HEPES-TMA buffer did not have any increase in the initial rate of chloride conductance when treated with the same in vitro activation conditions (14). Activation of protein kinase A in the I-A vesicles that are responsive to in vitro activation of conductive Cl⁻ transport may have produced an increase in 32P transfer to brush-border protein in molecular mass regions corresponding to the 130- and 23-kDa regions identified by differential labeling with fluorescein. There is no obvious correspondence of these molecular masses with the possible chloride channel proteins identified by Landry et al. (7) in ligand binding procedures using canine tracheal epithelium or by Finn et al. (6) in Necturus gall bladder. It is not clear why different identification procedures used on different tissue sources appear to involve proteins of different size in the conductive transport of Cl⁻ across apical membrane of exocrine tissues. Perhaps some indications of interactions with the cystic fibrosis transmembrane regulator (CFTR) protein or identity to CFTR may clarify the involvement of specific membrane proteins with exocrine chloride conductance.

The CFTR gene product has been reported to migrate with an apparent molecular mass of 130 kDa (20), so the current findings cannot be used to rule out a role for CFTR in conductive chloride transport. Precise identification of a membrane protein responsible for conductive transport of Cl⁻ in the apical membrane of exocrine tissues may require some combination of techniques for differential labeling, production of specific antibody, and isolation and functional reconstitution.

REFERENCES