Reconstitution of an Epithelial Chloride Channel

Conservation of the Channel from Mudpuppy to Man

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ABSTRACT We have previously shown that monoclonal antibody E12 (MAb E12), one of several such antibodies raised against theophylline-treated *Necturus* gallbladder (NGB) epithelial cells, inhibits the chloride conductance in the apical membrane of that tissue. Since chloride channels are critical to the secretory function of epithelia in many different animals, we have used this antibody to determine whether the channels are conserved, and in an immunoaffinity column to isolate the channel protein. We now demonstrate that MAb E12 cross-reacts with detergent-solubilized extracts of different tissues from various species by enzyme-linked immunosorbent assay (ELISA). Western blot analysis shows that this monoclonal antibody recognizes proteins of M_r 219,000 in NGB, toad gallbladder, urinary bladder, and small intestine, A6 cells, rat colon, rabbit gastric mucosa, human lymphocytes, and human nasal epithelial cells, and inhibits the chloride conductance in toad gallbladder, rat colon, and human nasal epithelium. Detergent-solubilized protein eluted from an immunoaffinity column and then further purified via FPLC yields a fraction (M_r 200,000–220,000) which has been reconstituted into a planar lipid bilayer. There it behaves as a chloride-selective channel (P_{Cl}/P_{Na} = 20.2 in a 150/50 mM trans-bilayer NaCl gradient) whose unit conductance is 62.4 ± 4.6 pS, and which is blocked in the bilayer by the antibody. The gating characteristics of this channel indicate that it can exist as aggregates or as independent single channels, and that the antibody interferes with gating of the aggregates, leaving the unit channels unchanged. From these data we conclude that the protein of M_r 219,000 recognized by this monoclonal antibody is an important component of an epithelial chloride channel, and that this channel is conserved across a wide range of animal species.

INTRODUCTION

Most epithelia contain chloride conductances, that is, pathways across which chloride ions are driven by the electrochemical potential gradient. In fact, net salt transport

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across most such epithelia is, at least in part, mediated by these pathways. Nonetheless, characterization of these conductances is far from complete, despite the now abundant evidence for the presence of chloride channels in epithelial tissues (Greger and Schlatter, 1984; Frizzell, Rechkemmer, and Shoemaker, 1986; Shoemaker, Frizzell, Dwyer, and Farley, 1986; Welsh and Liedtke, 1986; Reinhardt, Bridges, Rummel, and Lindemann, 1987; Bridges, Worrell, Frizzell, and Benos, 1989). Of interest is the fact that *Necturus* gallbladder (NGB) is the only tissue in which it has been shown that a large chloride conductance can be activated by an appropriate stimulus (a rise in cellular cAMP), but in which no such conductance can be detected under control conditions (Petersen and Reuss, 1983). Because of the critical role of the epithelial chloride channels in chloride secretion, and because of the large chloride conductance in the activated NGB, we decided to use this tissue as a model to characterize these channels. As previously reported (Finn, Tsai, and Falk, 1989), we raised monoclonal antibodies against theophylline-treated NGB epithelial cells; one of these antibodies, MAb E12, inhibits >80% of the cAMP-activated chloride conductance in NGB. In addition, Western blot analysis shows that a protein band of ~219 kD is one of the major proteins in NGB epithelium recognized by this antibody. Owing to the almost ubiquitous existence of chloride channels, we have used the antibody to examine its recognition of proteins from other species (by Western blot and ELISA), and have also studied its effects on the chloride conductance in a variety of tissues from diverse species. We now report that this monoclonal antibody recognizes similar antigenic determinants and fully blocks the chloride conductance in a wide variety of tissues; furthermore, we use the antibody as an affinity ligand to purify the channel and reconstitute it into planar lipid bilayers to study some of its functional characteristics.

MATERIALS AND METHODS

Animals and Cell Lines

Mudpuppies (*Necturus maculosus*) were purchased from Kons Scientific Co., Inc. (Germantown, WI) and toads (*Bufo marinus*) from Carolina Biological Supply Co. (Burlington, NC). Mudpuppies were kept in a refrigerated aquarium (10–15°C). The animals were pithed and the gallbladders, small intestines, or urinary bladders were removed. Rat colons were obtained from male Sprague-Dawley rats (375–500 g) killed by cervical dislocation. The colon of each animal was removed, opened longitudinally, and washed of contents with oxygenated Ringer solution. The epithelial cells of these tissues were removed by scraping for further preparation of soluble extracts. For electrophysiological studies, the intact epithelia were removed. A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 69th plating. The growth medium consisted of NCTC-135 (75%), distilled water (15%), and fetal bovine serum (10%). Cells were grown at 28°C in a humidified incubator gassed with 5% CO_2. The cells used for this study were between the 70th and 80th platings. Human lymphocytes were isolated from heparinized blood from healthy donors. Rabbit gastric mucosal membranes were a gift from Dr. John Forte of the University of California at Berkeley, and T84 cells, a cell line originating from human colon carcinoma (Dharmsathaphorn and Pandol, 1986; Halm, Rechkemmer, Schoumacher, and Frizzell, 1988) from Dr. Helen Berschneider of North Carolina State University.

Cell Preparations

Cell homogenates were prepared in ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM aprotinin, and 1 μM leupeptin), subjected to ultrasonication or nitrogen cavitation, solubilized in 1% Triton X-114 for 1 h at 4°C, and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and stored at −70°C. The protein concentration was determined using a Coomassie blue G-250 binding assay kit (Bio-Rad Laboratories, Richmond, CA).

Preparation of Monoclonal Antibodies

Monoclonal antibodies were raised as described in our previous report (Finn et al., 1989). Briefly, BALB/c mice were immunized with a nitrogen cavitate of theophylline-treated epithelial cells scraped from NGB. Sera from these animals bound to the epithelial cells of NGB as assessed by immunofluorescence microscopy and inhibited the theophylline-induced chloride conductance. Hybridomas were then prepared using standard techniques.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs were carried out on poly-L-lysine–coated microtiter plates (Costar Corp., Cambridge, MA). 1 μg/well of soluble extracts from different cell sources were added for 1 h at room temperature and incubated overnight at 4°C. This procedure was followed by washing three times with PBS, incubating with 1% gelatin in PBS for 1 h, then rewashing three times. PBS or a different dilution of antibody was added and incubated for 1 h at room temperature. After three washings with PBS, the plates were incubated for 45 min at room temperature with horseradish peroxidase–conjugated goat anti–mouse IgG and IgM (Tago, Inc., Burlingame, CA). The substrate for the peroxidase reaction was a solution of ortho-phenylene diamine dihydrochloride (0.04%) in citrate phosphate buffer (0.1 M Na citrate, 0.2 M Na_2HPO_4, pH 5.0) with hydrogen peroxide (0.012%). Optical density was read at 450 nm with a microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). For absorption studies, cell pellets (obtained from epithelia by scraping, nitrogen cavitation, and centrifugation at 10,000 rpm for 30 min) from different tissue sources were mixed end-over-end with diluted monoclonal antibody solution for 1 h at room temperature, then overnight at 4°C. The mixtures were centrifuged at 10,000 rpm for 20 min. The resulting supernatants were stored at −20°C. The same antibody solution not depleted by absorption was processed in a similar manner and used as a control.

Western Blot

Electrophoresis was carried out by standard methods (Laemmli, 1970) using 5% SDS-polyacrylamide and 10% 2-mercaptoethanol. Electrophoretic transfer of protein from gels to nitrocellulose paper (0.45 μm; Schleicher & Schuell, Inc., Keene, NH) was done by standard methods, and transferred protein was visualized by staining the paper with amido black. The nitrocellulose strips were blocked for 4 h at room temperature with TBS (1.5 M NaCl, 0.5 M Tris, pH 7.3) containing 0.5% Tween 20, washed, and then incubated overnight at 4°C with primary antibody. They were subsequently washed and incubated with peroxidase conjugated goat anti–mouse IgG and IgM for 3 h. The nitrocellulose strips were then washed five times and the peroxidase activity was detected with a substrate solution made of H_2O_2 (0.015%), 4-chloro-1-naphthol (0.05%), and methanol (16.5%).

Studies on Isolated Epithelia

Electrophysiological studies were performed on a number of epithelia. In the case of *Necturus* and toad, intact gallbladders were removed and placed, mucosal side up, across a 0.5-cm^2

opening between two lucite chambers. Both sides of the tissue were perfused with a solution containing (in mM): 95 NaCl, 10.0 $NaHCO_3$, 1.2 K_2HPO_4, 0.1 KH_2PO_4, 1.0 $MgCl_2$, and 2.0 $CaCl_2$; it was gassed with a mixture of 99% O_2 and 1% CO_2 and had a pH of ~7.7. Transepithelial potential was measured with silver–silver chloride electrodes that contacted the serosal and mucosal solutions via Ringer-filled bridges. Current was passed across the epithelium through ring-shaped platinum–iridium electrodes placed directly in the mucosal and serosal solutions. Apical membrane potential was measured with glass microelectrodes filled with 0.5 M KCl with resistances of 100–170 MΩ. All electrodes were connected to a current–voltage clamp device that was used to pass transepithelial constant current pulses for the purpose of measuring the fractional apical resistance ($= R_a/[R_a + R_b]$, where R_a is the apical and R_b the basolateral membrane resistance), determined as the ratio of the current-induced voltage deflection at the apical membrane to that across the entire tissue. The desired voltage outputs of the clamp device were displayed on an oscilloscope and sampled by a computer. Cells were impaled and the apical membrane potential was followed during rapid isosmotic changes in mucosal chloride concentration (from 101 to 10 mM or the reverse, replaced by cyclamate). In the case of the NGB, this procedure was followed by the measurement of the apical membrane potential change under similar conditions in the presence of 0.25–0.75 mM serosal theophylline. We then removed the mucosal solution, put an aliquot of a given monoclonal antibody on the mucosal surface, and allowed it to remain there at room temperature for 15–30 min. We then repeated the change in medium chloride and determined the magnitude of the membrane potential change. This change (depolarization, accompanied by a rise in the relative resistance of the apical membrane when the chloride concentration was decreased, and a hyperpolarization and decrease in resistance when the concentration was increased) was used as a semiquantitative estimate of the conductance of the apical membrane to chloride. To study the effects of the antibody on the relative conductance to other ions, we impaled a series of cells with 10 mM chloride on the mucosal side, and in each case then changed to a 10 mM sodium solution (substitution with *N*-methyl-D-glucamine, NMDG), and finally to a 97.5 mM potassium (substituting K for NMDG) solution, always with 10 mM chloride. We then replaced the medium with normal Ringer, and after all potentials and resistances had returned to control values we added antibody as above and repeated the experiments on another series of cells. Although not shown here, the chloride-dependent change in membrane potential appears within 5 s after the addition of theophylline and remains unchanged for at least 3 h. Furthermore, removal of theophylline (which causes total disappearance of the chloride-dependent potential within 30 s) followed by readdition of the drug at the same concentration leads to the development of a chloride-dependent potential of a magnitude indistinguishable from that seen after the first addition (data not shown).

For the study of rat colon, tissues were excised and the subepithelial muscle layers were removed by blunt dissection. The epithelia were mounted in lucite half-chambers with 0.5-cm^2 apertures and bathed in rat Ringer solution containing (in mM): 115 NaCl, 25 $NaHCO_3$, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose. The temperature was controlled at 37°C. The bathing solutions were connected via Ringer–agar bridges to calomel electrodes in order to measure the electrical potential difference across the preparation. Current was passed via Ag-AgCl electrodes connected to the bathing solution via Ringer–agar bridges. The potential was clamped to zero and the resulting current (the short-circuit current, I_{sc}) was determined by an automatic voltage clamp (World Precision Instruments, Inc., New Haven, CT).

Finally, nasal epithelial cells from excised nasal polyps obtained from otherwise normal patients were grown to confluence on a collagen matrix as previously described (Yankaskas, Cotton, Knowles, Gatzy, and Boucher, 1985; Willumsen, Davis, and Boucher, 1989a), and placed in the chamber as described above for gallbladder. They were perfused with a solution

identical to that used for the rat experiments described above. Tissues were studied as with the *Necturus* and toad except that the mucosal chloride concentration was varied only between 60 and 105 mM.

Affinity Chromatography

Antibodies were produced in mice ascites fluid by the method of Brodeur et al. (Brodeur, Tsang, and Larose, 1984). Briefly, ascites was produced by injecting hybridoma cells ($\sim 10^6$ cells/mouse) into the peritoneal cavities of BALB/c mice (5–8 wk old) pretreated with pristine. The mice were observed for abdominal swelling, and fluid was collected in sterile centrifuge tubes and centrifuged at 10,000 rpm for 10 min. Supernatant was collected and stored at −20°C.

The isotype of the monoclonal antibody was determined to be of the IgG 2b subtype by radial immunodiffusion using class- and subclass-specific reagents (The Binding Site Ltd., Birmingham, UK). A column was then packed with gamma-bind G-agarose (Genex, Gaithersburg, MD) and washed with loading buffer (10 mM Na_2HPO_4, 150 mM NaCl, 10 mM EDTA, and 0.005% NaN_3, pH 7.0). Ascites fluid was dialyzed against loading buffer and applied to the column. The column was washed with loading buffer until the absorbance of the eluate at 280 nm approached background. The IgG was eluted with 0.5 M acetic acid (pH 3.0) and the eluate was neutralized immediately with 2 M Tris.

CNBr-activated Sepharose 4B beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) were swollen in and washed with a 100-fold volume of 1 mM HCl on a sintered glass filter at room temperature. The resulting loosely packed beads were mixed together with IgG in coupling buffer (0.5 M NaCl containing 0.2 M $NaHCO_3$, pH 8.3). The mixture was rotated end-over-end overnight at 4°C and unbound IgG was washed away with coupling buffer; the remaining active groups were blocked with Tris-HCl buffer (0.1 M, pH 8) for 2 h at room temperature. Unreacted material was removed by washing and the resulting adsorbent was washed with PBS and stored at 4°C.

For chromatography, the immunoaffinity adsorbent was added to solubilized NGB cell extract. The mixture was incubated for 16 h at 4°C and transferred into a 10-ml plastic column and the beads were allowed to settle. The column was rinsed with PBS containing 0.1% Triton X-100 and eluted with elution buffer (0.2 M KSCN containing 0.5 M NaCl, 0.1% Triton X-100, and 0.5 M imidazole, pH 7.5). Fractions were dialyzed against PBS for 16 h using 10-mm-diam Spectra/Por dialysis membranes (14 kD cut-off; Spectrum Medical Industries, Los Angeles, CA).

FPLC

A 200-μl aliquot of immunoaffinity-purified protein in PBS containing 0.1% Triton X-100 was fractionated on a FPLC Superose 12 preparative column (100 ml) at a flow rate of 15 ml/h at 4°C. 1.0-ml fractions were collected, concentrated by Centricon 30 microconcentrators (Amicon Corp., Danvers, MA), and tested by incorporation into planar lipid bilayers.

Reconstitution Studies

The reconstitution protocol was basically as previously described (Alvarez, 1986; Rosenberg, Hess, and Tsien, 1988), with the exception that we added solubilized protein directly to the *cis* solution according to the method used by Meissner and his colleagues (Liu, Lai, Rousseau, Jones, and Meissner, 1989) to characterize the purified calcium release channel (M_r 400,000) from skeletal muscle sarcoplasmic reticulum. Planar lipid bilayers were formed from a mixture of palmitoyl-oleoyl phosphatidyl ethanolamine and palmitoyl-oleoyl phosphatidyl serine (3:1) (Avanti Polar Lipids, Inc., Birmingham, AL) in *n*-decane. We painted them across a 150-μm-

diam hole in a polyvinylidene difluoride cup set into a Teflon block, creating two chambers of ~3 ml volume each. Electrical contact was made through 0.5 M KCl–agar bridges to silver–silver chloride electrodes in adjacent wells for use in a two-electrode voltage-clamp system (model 3900; Dagan Corp., Minneapolis, MN).

Lipid bilayers had resistances of 70–150 GΩ and capacitances of 90–140 pF. At the time of bilayer formation, both solutions contained 50 mM NaCl and 10 mM HEPES, pH 7.0. Aliquots of the protein solution eluted from the immunoaffinity column or from the FPLC fractionation were added to the *cis* chamber, and after incorporation and recording, the concentration of sodium chloride in the *cis* chamber was changed to a final value of 150 mM.

Bilayers were clamped at voltages from −100 to +100 mV, with the voltage defined as *trans* − *cis* (ground). At positive holding potentials, currents flowing from *trans* to *cis* represent "outward" currents and are shown as upward transitions in the recordings. Currents were recorded at a bandwidth of 2,000–3,000 Hz and stored on a video cassette recorder; subsequently they were filtered at 200 Hz, digitized at 1,000 Hz, and analyzed.

RESULTS

ELISA

To determine the cross-reactivity of MAb E12, ELISA was carried out using soluble extracts from diverse tissues as antigen sources. As shown in Fig. 1, this antibody cross-reacts with toad gallbladder and small intestine, rat colon, and *Torpedo californica* electric organ. In similar studies (data not shown) the antibody also reacted with A6 cells, human lymphocytes, and *Necturus* small intestine. We also examined the reactivity of antigen with antibody preabsorbed with tissue extracts different from the target antigen (NGB). As shown in Fig. 2, the effect of antibody preabsorbed with A6 cells, rat colon, human lymphocytes, *Necturus* intestine, toad gallbladder, and toad small intestine is not different from the effect of antibody preabsorbed with the target antigen, indicating that all of these tissues contain epitopes in common with those in NGB. Preabsorption of antibody with myosin, fibronectin, or human serum did not significantly change reactivity against NGB (data not shown). Finally, we compared, at a single antibody dilution, the reaction of the monoclonal antibody with a series of cells of human origin to that with the same amount of antigen from NGB. Fig. 3 shows that the antibody also recognizes both epithelial (derived from colon, trachea, or nasal epithelium) and nonepithelial (lymphocytes and neutrophils) cells.

Western Blot Analysis

Antigens from cell sources from different species were electrophoresed, transferred onto nitrocellulose paper, and subjected to immunodetection (Fig. 4). MAb E12 cross-reacts with proteins of M_r 219,000 in toad gallbladder, toad urinary bladder, A6 cells, rat colon, rabbit gastric mucosa, human lymphocytes, and human nasal polyp cells, and with proteins of M_r 219,000 and 190,000 in toad small intestine. On the other hand, MAb G6, a monoclonal antibody to NGB subepithelial smooth muscle cells that has no effect on the chloride conductance (Finn et al., 1989), recognizes a protein band of ~350 kD in NGB. Although not shown here, neither antibody recognizes any antigen (by ELISA or Western blot) from bovine serum albumin, human serum, or human collagen.

Electrophysiological Studies

The effect of MAb E12 on chloride conductance in NGB is shown in Fig. 5. In the absence of antibody ("control"), reduction in mucosal solution chloride concentration leads to a gradual hyperpolarization of the apical membrane potential and no significant change (from 0.65 to 0.68 in this case) in fR_a. The hyperpolarization is apparently due to changes in basolateral membrane E_{Cl} and sodium transport rate (Stoddard and Reuss, 1989), but in any case is in the direction opposite to that expected for an apical chloride channel. Immediately after the addition of theophylline there is a marked depolarization of the membrane potential and a decrease in fR_a as previously described (Petersen and Reuss, 1983); these changes persist for at

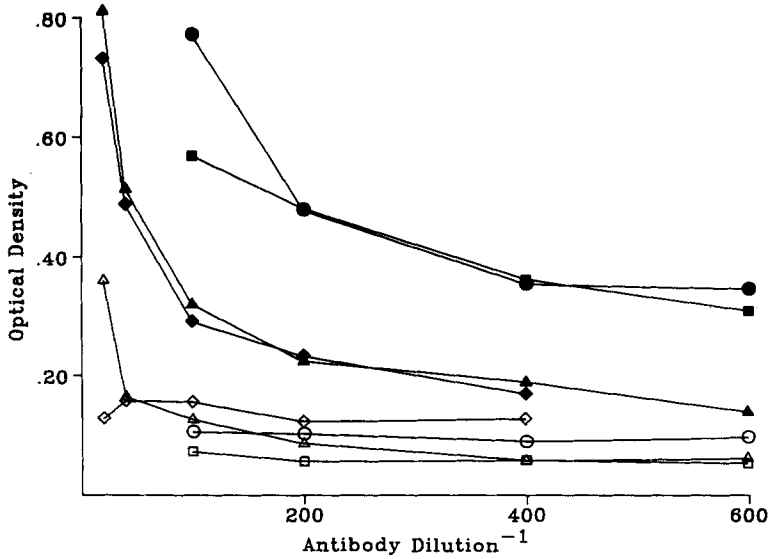

FIGURE 1. ELISA. The optical density of the reaction product of horseradish peroxidase is plotted against the reciprocal of the antibody dilution. The solid symbols show data from unabsorbed antibody, the open symbols from antibody preabsorbed by the target antigen. *Circles,* toad gallbladder; *squares,* toad small intestine; *triangles,* rat colon; *diamonds, Torpedo californica* electric organ.

least several hours as long as the agonist is present (Finn et al., 1989). The reduction in chloride concentration now results in an immediate further depolarization and an increase in fR_a (from 0.27 to 0.39), confirming the presence of a chloride conductance in the apical membrane. After incubation with antibody, the chloride conductance is no longer present; that is, reduction in chloride concentration leads again to hyperpolarization without a significant change in fR_a.

Although not shown here, there is no effect of the antibody in the absence of theophylline, indicating that there are no obvious effects of this material on baseline resistance and conductances. To test this further, we performed experiments in which we measured the effects of the antibody on the apparent potassium and sodium

conductances of the apical membrane. In these studies, we altered the concentration of each of these ions before and after adding antibody (as noted in Materials and Methods, the concentration of chloride in the solution is kept at 10 mM). As shown previously (Reuss and Finn, 1975*a*, *b*), the apical membrane under control conditions is dominated by a potassium conductance, and there is a small conductance to sodium. In the present experiments (3 tissues, 14 cells impaled before antibody and 14 after antibody), the mean change (hyperpolarization, consistent with the presence of a sodium conductance) in the apical membrane potential upon lowering the

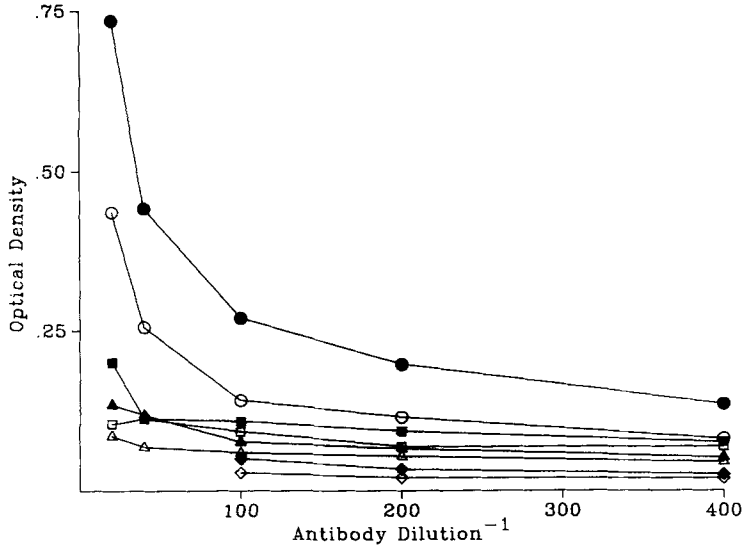

FIGURE 2. ELISA. In each case, the antigen in the wells was an extract from NGB epithelial cells. In the data shown by solid circles, antibody was unabsorbed. In each of the other sets of data shown, the antibody was first absorbed with antigen from another tissue extract, and the supernatant from this incubation (see Materials and Methods) was added to each well at the dilution shown. There was significant absorption of the antibody in each of these cases, so that the optical density of the reaction product was less than control (i.e., with unabsorbed antibody, solid circles) at every antibody dilution, indicating the sharing of common epitopes among these antigens. *Open circles,* A6 cells; *filled squares,* rat colon; *open squares,* NGB; *filled triangles,* human lymphocytes; *open triangles, Necturus* intestine; *filled diamonds,* toad gallbladder; *open diamonds,* toad intestine.

mucosal sodium concentration was 14.4 ± 1.3 mV (from 65.5 ± 1.2 to 79.9 ± 1.5) before antibody and 17.6 ± 1.8 mV (from 67.0 ± 2.0 to 84.6 ± 2.5) after antibody; in the same cells, the mean change in the apical membrane potential upon raising the potassium concentration was (a depolarization of) 45.4 ± 2.0 mV before antibody and 49.5 ± 1.6 mV after antibody. None of the antibody-induced changes was significant. There were also the expected changes in fractional resistance (increase upon reduction in sodium, decrease upon the rise in potassium concentration) as previously reported; again, these changes were no different before or after antibody.

Thus the antibody has no effect on the potassium or sodium conductances in the NGB in the same concentrations at which it abolishes the chloride conductance induced by the addition of theophylline.

MAb E12 also inhibits the chloride conductance in the toad gallbladder. Three of the four tissues studied showed no chloride-dependent membrane potential change in the absence of theophylline (as in all NGB); all of these responded to 0.5 mM theophylline with an increase in the apical chloride conductance. The other one had a large chloride-dependent membrane potential change even in the absence of theophylline, and results from this tissue are shown in Fig. 6. In all four cases, the antibody completely blocked the change; the mean values were 14.2 ± 1.1 mV (4 bladders, 17 cells, before antibody) and 0.3 ± 0.6 mV (18 cells, after antibody).

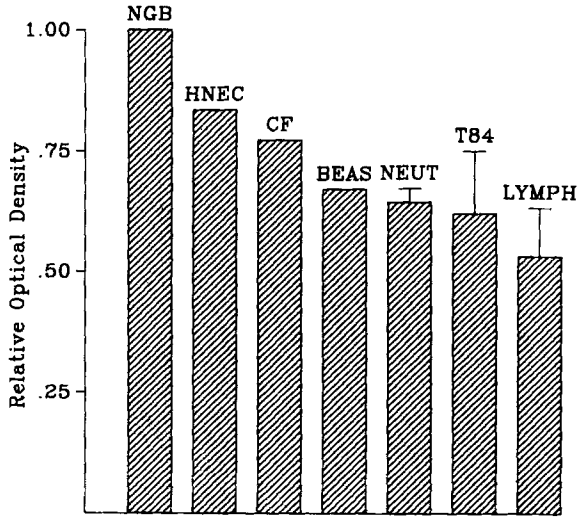

FIGURE 3. ELISA on cells of human origin. In these five studies, a single dilution of antibody (1:20) was used, and each assay was done in duplicate. To adjust for variability from day to day, data are presented as the ratio of the optical density (OD) of the sample to that of NGB antigen whose reactivity with MAb E12 was tested in each experiment. Both NGB and the test OD were corrected for the background OD observed with PBS alone. All values were at least twice the background level, with a mean ratio of sample OD/background OD of 9.0 ± 1.4. One study each (in duplicate) was done with human nasal epithelial cells (*HNEC*), a cell line from human trachea originally obtained from a patient with cystic fibrosis (*CF*), and a cell line from normal human trachea (*BEAS*), and three studies each were done with human neutrophils (*NEUT*), T84 cells, and human lymphocytes (*LYMPH*). Bars represent SEM.

To test the influence of MAb E12 on the chloride conductance in rat colon, I_{sc} was measured. Fig. 7 shows the results of these studies. The control I_{sc} is carried by the net inward (i.e., absorptive) movement of sodium, and Iloprost, an analogue of prostacyclin, increases the I_{sc} by stimulating outward chloride transport, presumably via the activation of apical chloride channels (Chang, Brown, Wang, Browning, Hardcastle, Hardcastle, and Sanford, 1977; Donowitz, Cheng, and Sharp, 1986; Halm and Frizzell, 1986). As seen in the figure, MAb G6 has no effect on the increase in I_{sc} in response to the agonist, whereas MAb E12 completely prevents this stimulatory response.

The effect of MAb E12 on the chloride conductance in cultured human airway cells was also measured. As shown in Fig. 8, and similar to studies previously reported

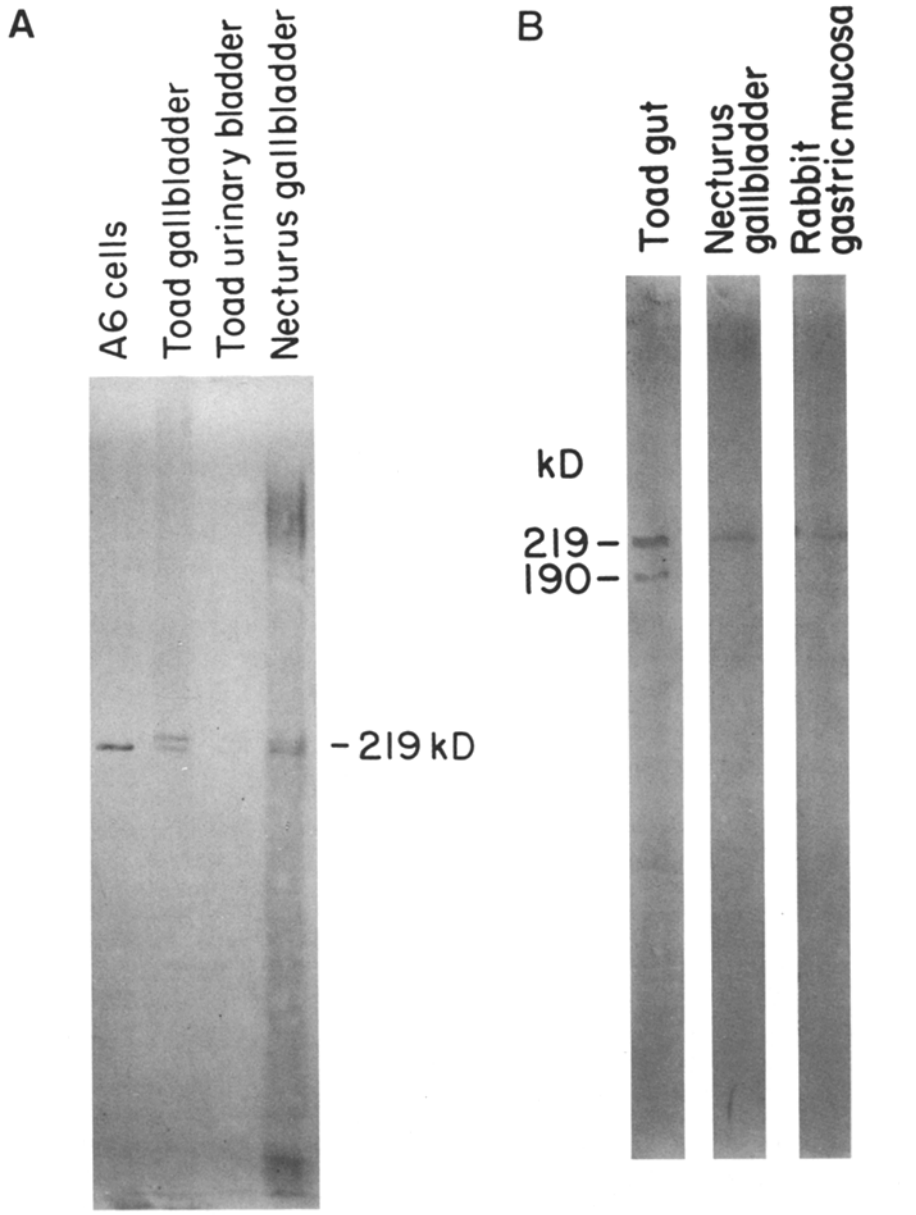

FIGURE 4. Western blot analysis of cell homogenates from several different tissue sources. In each case, we used NGB as a positive control. All blots are performed with a strip of molecular weight markers; they are omitted from the figures. (*A*) Each of the tissues shows one or two bands at a molecular mass of ~219 kD, essentially identical to that recognized in NGB. (*B*) Toad small intestine homogenate was prepared from scraped cells, and rabbit gastric mucosa is an apical membrane homogenate. As in *A*, the antibody recognizes a band at 219 kD (and the additional band in toad intestine). (*C*) Human lymphocytes and rat colon cells share a 219-kD band recognized by the antibody. (*D*) There is a similar band in another tissue of human origin. The nasal polyp cells were from primary cultures obtained from an otherwise normal patient.

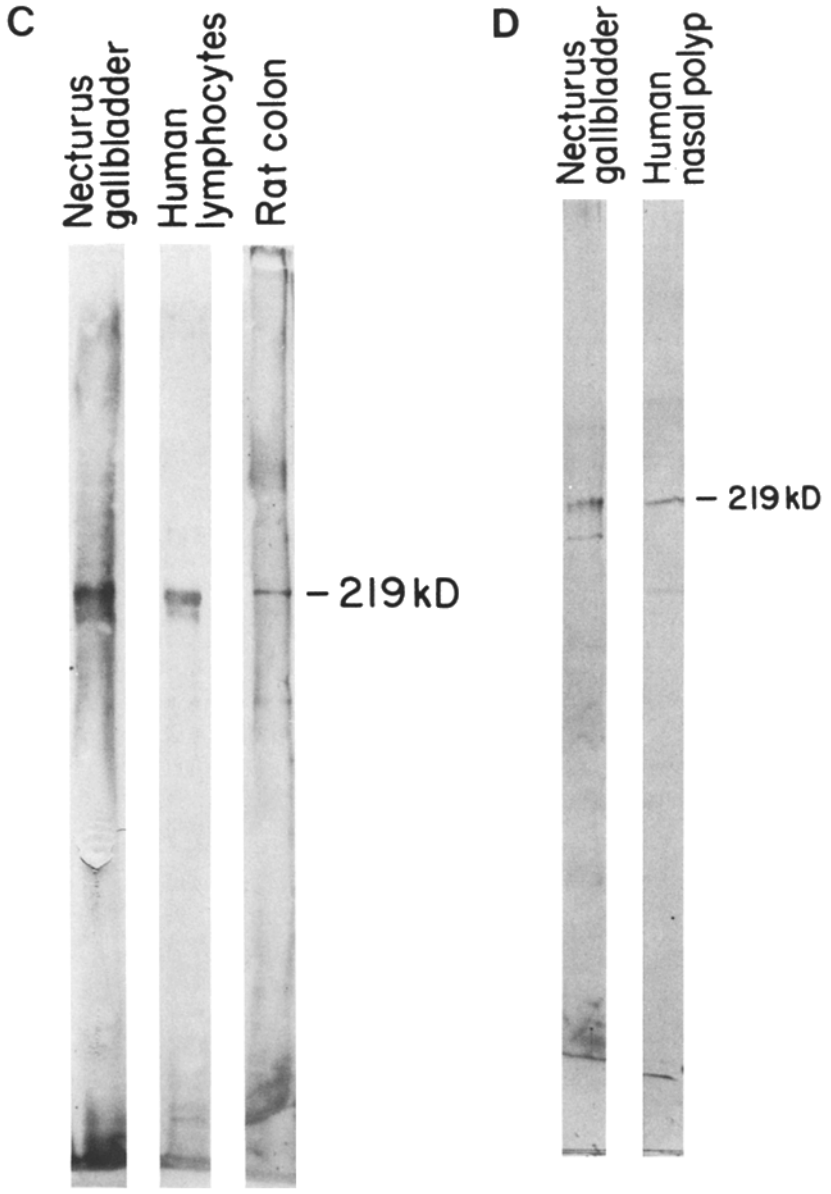

FIGURE 4. (continued)

(Willumsen, Davis, and Boucher, 1989*b*), the increase in mucosal chloride concentration from 60 to 105 mM leads to hyperpolarization and a slight decrease in fractional apical resistance, indicating the presence of a chloride conductance. After exposure to MAb E12, the response to this change in chloride concentration is completely lost. In another preparation, the cells were first exposed to MAb G6, and several

impalements showed no inhibition of the conductance. After subsequent exposure to MAb E12, there was complete inhibition of the response to the change in chloride concentration.

Reconstitution Studies

Immunopurified protein was prepared by passing detergent extracts of NGB epithelial cells over an immunoaffinity column as described in Materials and Methods. An example of an immunoblot of the crude extract and the purified column eluate is shown in Fig. 9. It is evident that a band of molecular weight identical to that seen in

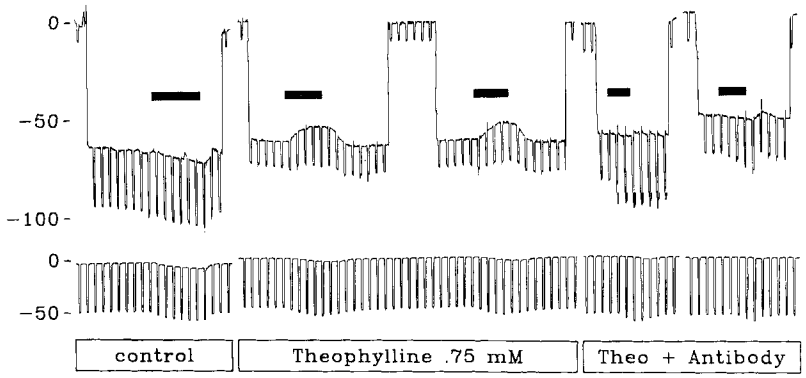

FIGURE 5. Inhibition by specific antibody of NGB chloride conductance. All traces are from a single tissue. The upper traces show the apical membrane potential observed during impalements of five cells under different conditions, and the lower traces show the simultaneously obtained transepithelial voltage values. The downward deflections at ~2-s intervals represent the responses to transepithelial constant current pulses, and allow one to calculate the fractional apical resistance, fR_a. The large downward deflection at the beginning of each upper trace is due to the impalement of a cell. The solid bars represent the times during which the chloride concentration in the mucosal bathing medium was reduced from 101 to 10 mM. About 30 s before the start of the second impalement, theophylline was added to the serosal bathing medium. After obtaining several similar impalements, the monoclonal antibody was placed on the mucosal surface for 30 min and washed off and further impalements were made. There was no significant effect of the antibody on transepithelial resistance. As shown, restoration of normal [Cl^-] often leads to a short-lived overshoot of the membrane potential.

the crude material is eluted from the column. Because nonimmunoreactive protein could remain in this fraction, further purification by gel filtration was done, as described below.

To determine if the immunoaffinity column eluate contained a protein that could function as a chloride channel, aliquots of the partially purified material were added to the *cis* side of preformed planar lipid bilayers. The bilayers were voltage-clamped at −60 mV and the current was monitored continuously. In 58 of 66 attempts to date, using three separate identically prepared immunoaffinity columns and six different sets of identically prepared cell homogenates, we recorded channel activity. The

addition of concentrated NaCl to the *cis* chamber (to 150 mM final concentration, shifting E_{Na} to +27.5 mV and E_{Cl} to −27.5 mV) revealed that the channel was highly selective for chloride over sodium (see below). In all cases, the channel behavior was extremely complicated, with many apparent multiple conductance substates and dramatic shifts in channel gating.

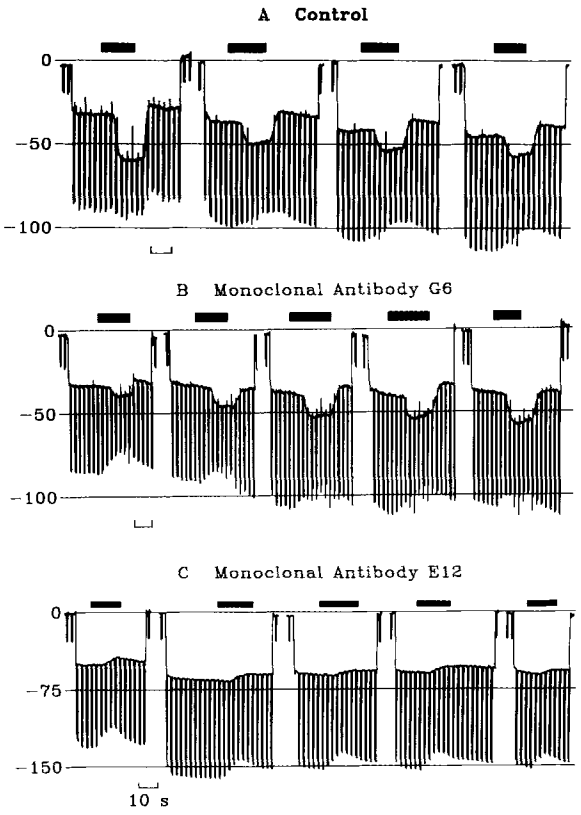

FIGURE 6. Inhibition by specific antibody of toad gallbladder chloride conductance. All traces are from a single tissue, and each one represents an impalement of a single cell. The top row shows the apical membrane potential observed during impalements of four cells under control conditions. The notation is as in Fig. 5 with the exception that in this experiment the chloride concentration was changed during the times indicated by the bars from 10 to 101 mM. Note that in the absence of antibody the rise in chloride concentration results in an immediate hyperpolarization and a large decrease in the voltage deflection across the apical membrane. There was almost no change in the deflection across the whole tissue, so that fR_a is markedly reduced. The combination of the hyperpolarization and the decrease in fractional resistance confirm the presence of a chloride conductance in the apical membrane. After obtaining several similar impalements, MAb G6 was placed on the mucosal surface for 30 min and washed off. The second row of impalements was then obtained; the results are not significantly different from those in the first row, confirming that this antibody has no effect on toad gallbladder apical membrane chloride conductance. MAb E12 was then placed on the mucosal surface for a similar period and washed off by the superfused solution. Subsequent impalements, as exemplified by the bottom row, indicate that the chloride conductance has completely disappeared; that is, a rise in the chloride concentration leads only to a slow depolarization (and no significant change in fR_a).

An example of the type of channel behavior we recorded is shown in Fig. 10*A*. In this and all subsequent figures showing channel activity the NaCl gradient, in millimolar, is 150/50 (*cis/trans*). The most prominent transition was ~10 pA at the −60 mV potential shown, but many other transitions are also apparent (for example, in the fourth trace). Analysis of these smaller transitions with an expanded time base

shows that they are well-resolved transitions to multiple conductance levels, as shown in Fig. 11. Such transitions occur from virtually all of the states shown; for instance, they are seen from the most closed state shown in Fig. 10 *A* during much of the fourth trace, but similar transitions occur also from much more open states, as seen at the end of the first trace.

The incorporation of a large conductance composed of smaller substates was common. In most cases, concerted gating transitions resulting in closing to the "bare bilayer" current level were extremely rare. However, smaller transitions from almost all conductance levels to another substate were readily recorded. Examples of the smallest of such transitions are shown in Fig. 12. In both experiments, incorporation

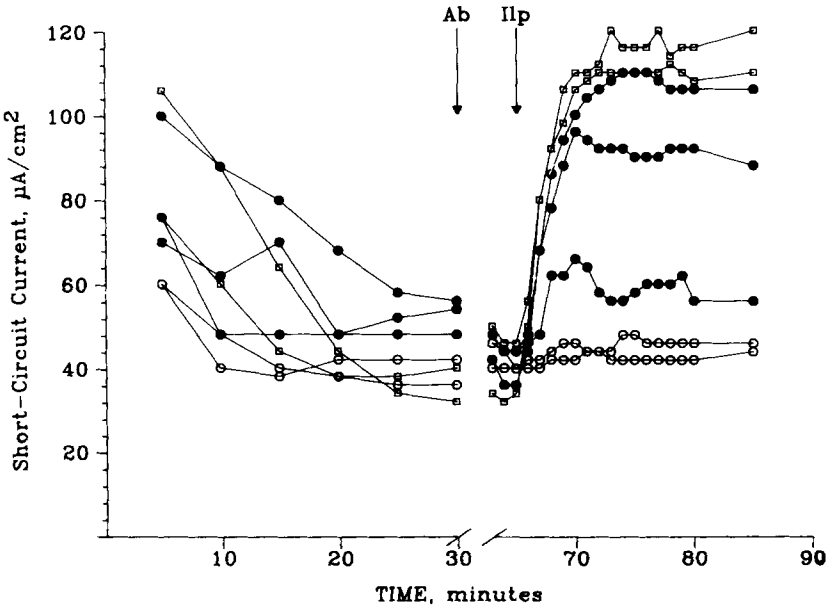

FIGURE 7. Inhibition of rat colon chloride channels. Short-circuit current was allowed to reach a stable value. At that time (*arrow Ab*), either nothing (control, *solid circles*), control monoclonal antibody (MAb G6, *open squares*), or active monoclonal antibody (MAb E12, *open circles*) was added to the mucosal bathing solution. 30 min later, antibody was washed off and Iloprost (final concentration 10^{-8} M) was added to the serosal solution of all tissues (*arrow Ilp*).

was signaled by a sudden increase to a large conductance level, and the "unitary" events were recorded after the elevation of the NaCl concentration on the *cis* side. In 11 experiments, the unitary conductance was 62.0 ± 4.6 pS and the reversal potential was −24.3 ± 0.8 mV, indicating a 20-fold selectivity for chloride over sodium.

To determine if the chloride channels recorded were sensitive to MAb E12, we added the antibody (1:100 dilution) to both sides of the bilayer. In every experiment (15 of 15 attempts to date) there was a dramatic shift to lower current levels. An example of this effect is shown in Fig. 10 *B*. Within 30 s after the addition of antibody, there is a clear shift away from the largest conductance level in favor of a

nearly fully closed state with some transitions to intermediate substates; after a short time, in each experiment, the large transitions became rare and the mean conductance decreased markedly. These effects are quantitated in the amplitude histograms shown in Figs. 13 and 14. In both experiments, a similar effect is seen: a reduction in the mean current and a decrease in the frequency of large transitions. In these experiments, it was possible to examine the records at high resolution to obtain current–voltage data from unitary transitions before and after antibody addition. The results shown in Fig. 15 indicate that neither the apparent unitary conductance nor the reversal potential was altered by the addition of antibody.

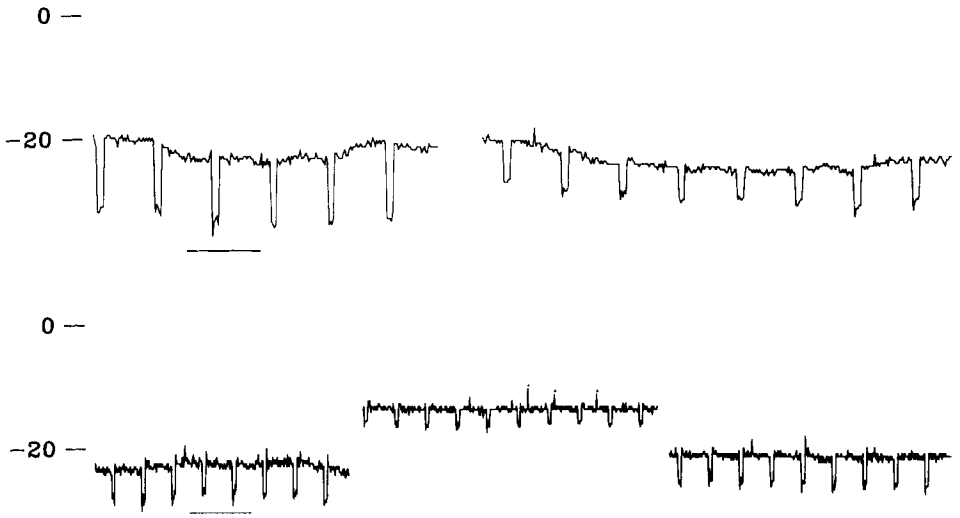

FIGURE 8. Inhibition of chloride conductance in cultured human airway cells. Traces begin with the electrode already in the cell. In each case, membrane potential and fR_a had been constant (within ± 10%) for at least 30 s before the change in the apical medium; the chloride concentration in that solution was raised from 60 to 105 mM just before the beginning of the trace, and remained at that concentration for 30 s. The slowness of the response in comparison to that shown in Fig. 5 is due to the slow perfusion necessary to maintain the impalements. The top two traces are from cells studied under control conditions, and the bottom three traces are from cells in the same preparation after exposure to MAb E12 for 30 min. The horizontal bars represent 10-s periods; the top one is appropriate for both control records, the bottom one for the three records in antibody-treated cells.

The recordings just described were obtained from material eluted from the immunoaffinity column. Although this material contained only one high molecular weight protein visible by Western analysis, we wished to confirm its size and purity by another method. Although unlikely, it is possible that our reconstituted channel was due to a low-abundance contaminant that uniformly coprecipitates from the immunoaffinity column. The method we chose was gel filtration analysis using FPLC technology. As shown in Fig. 16, we obtained one major protein peak at M_r ~220,000. Material from the center of the peak (fractions 34 and 35) was pooled, concentrated, and incorporated on two occasions into planar lipid bilayers as

described above. The observed channels behaved in a manner that was indistinguishable from those described above. The unitary conductances were 31.5 and 23.9 pS, and the reversal potentials −21.4 and −24.1, respectively. These data strongly indicate that, if a contaminant is our reconstituted channel, it must have a molecular weight in the 220,000-D range.

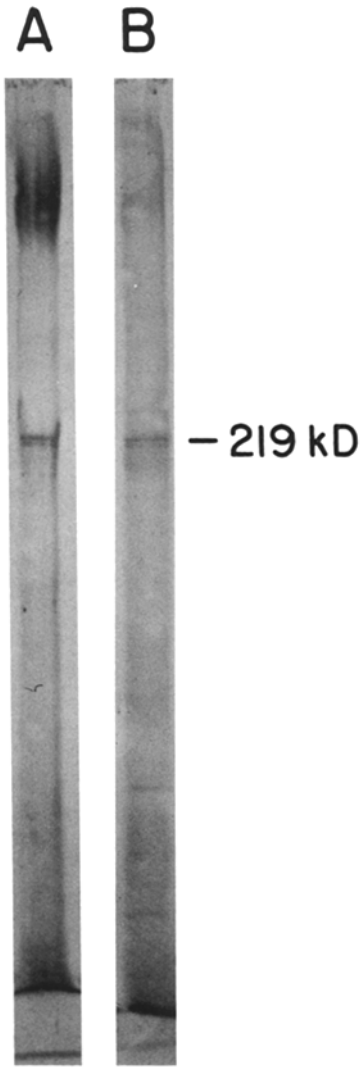

FIGURE 9. Western blot analysis of eluate from immunoaffinity column (*A*) compared with crude material introduced into the same column (*B*). The major band at 219 kD is essentially unchanged by the column and is the only one recognized in either trace. Though not shown here, a similar blot done on the material that washed through the column (that is, material that was not held back by antibody binding) showed no immunoreactivity.

We did a number of control experiments to be certain that the recorded channels indeed represented incorporation of the purified protein. First, multiple additions of the elution buffer alone, or of Triton X-114 or X-100 alone at concentrations of 0.05–2%, yielded no channels, nor have experiments in which we added aliquots of the wash-through from the immunoaffinity column (material that does not bind, and

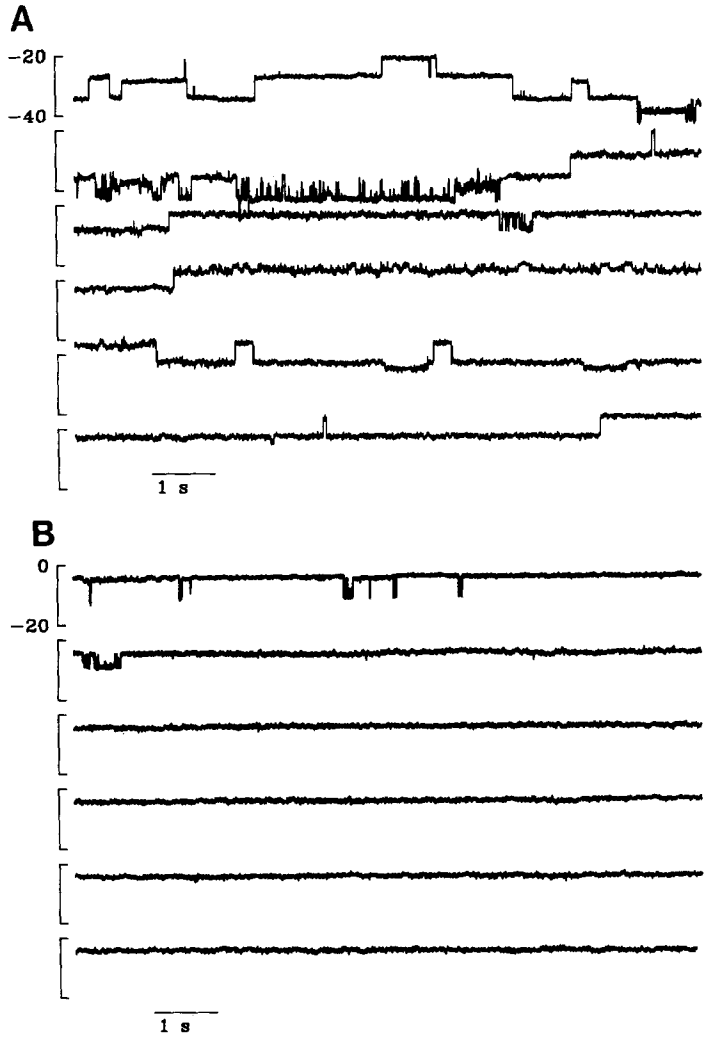

FIGURE 10. Reconstituted channel. Continuous traces of 1-min duration, obtained at a transmembrane voltage of −60 mV; thus, channel openings are downward transitions. In this and all traces of reconstituted channels, there is a 150/50 mM *cis/trans* NaCl gradient. (*A*) Control. In each trace the ordinate shows the current in picoamperes; only the first trace is marked for ease in reading, but all ordinate axes are identical. The current never approaches a fully closed level, and there are many transitions to various open states. (*B*) Effect of antibody. This record was started ~30 s after antibody (1:100 dilution) was added to both sides of the bilayer. As in *A*, the ordinate is marked only in the top trace, but applies to all traces. The gain is the same as in *A*, but the offset is different.

which contains proteins from the gallbladder cells other than those that bind to the column). In addition, we have tried on four occasions to incorporate immunoaffinity-purified myeloperoxidase (in PBS containing 0.1% Triton X-100), a protein of 120,000–150,000 mol wt, but have not seen any channel behavior. Finally, we

removed an aliquot from one cell homogenate preparation that had already been successfully reconstituted as chloride channels in 15 of 15 attempts, and placed it in a boiling water bath for 5 min. We then added aliquots of this material to the reconstitution system exactly as with the unaltered homogenate. In three separate attempts no channels could be incorporated, indicating that the protein was probably denatured and therefore would not form channels, and that the rest of the detergent-containing solution could not by itself cause channel formation.

Thus, the reconstitution experiments show that a protein eluted from the immunoaffinity column with a high molecular mass (219 kD) as seen by Western blot and confirmed by FPLC, reproducibly incorporates as a channel that is highly selective for

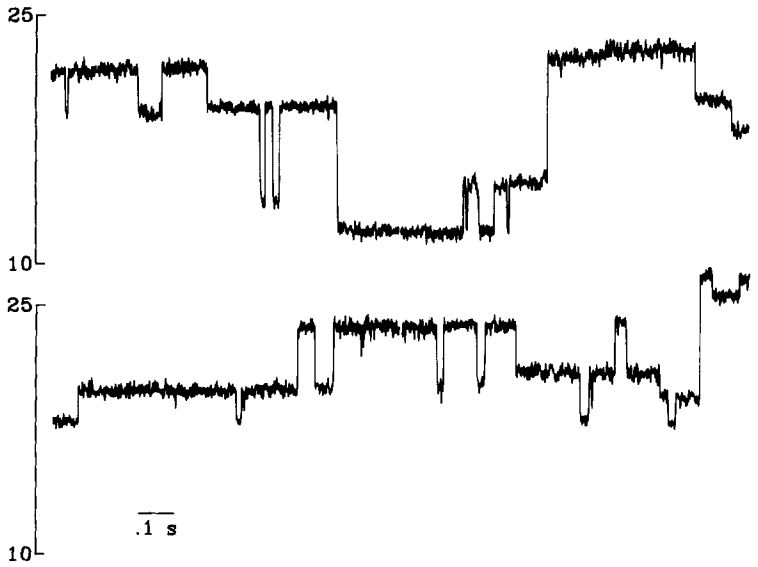

FIGURE 11. Currents at 0 mV. This is a high resolution trace of 4 s of data obtained under control conditions from the experiment shown in Fig. 10. Channel openings are upward. Transitions from this short section and from the rest of the record at this potential vary from 1.5 to 7.5 pA, with intermediate transitions of 2, 2.5, 3, 4, and 5.5 pA. The ordinate is in picoamperes.

chloride over sodium, always seems to exist in multiple states, and is almost completely blocked by antibody.

DISCUSSION

Chloride channels are common to both excitable and nonexcitable cells, and much is known about their electrical characteristics in many systems. In epithelia, many such channels have been demonstrated either by patch-clamping, for example, of airway (Frizzell et al., 1986; Shoemaker et al., 1986; Welsh and Liedtke, 1986), kidney (either native [Light, Schwiebert, Fejes-Toth, Naray-Fejes-Toth, Karlson, McCann, and Stanton, 1990] or in culture [Nelson, Tang, and Palmer, 1984]), or gallbladder

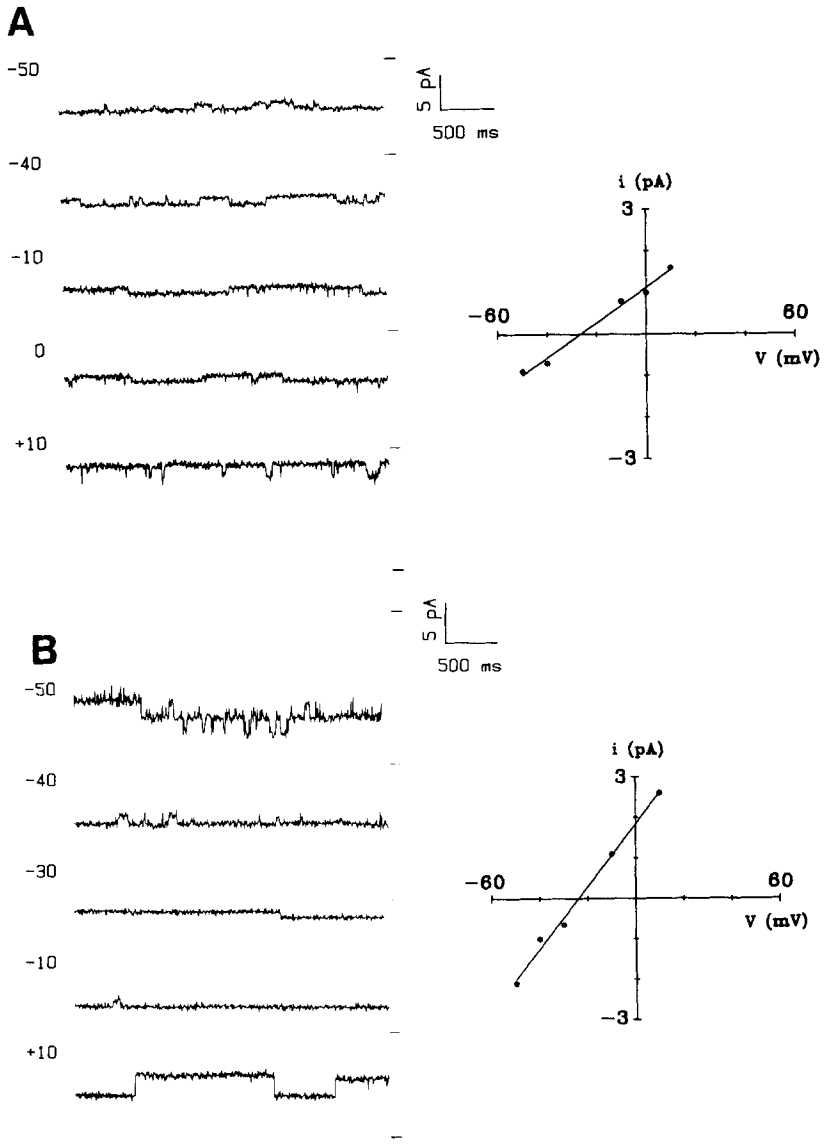

FIGURE 12. Reconstituted channels, showing the smallest detectable (unitary) transitions from two separate experiments. At each clamped voltage, the zero current level is marked at the right of the trace, and in no case was there a transition to the "bare bilayer" level. In both cases the complete records also showed larger transitions similar to those in Figs. 10 and 11. No antibody was present. (*A*) Conductance is 42 pS and the reversal potential is −26.5 mV. If the total conductance were composed of identical 42-pS channels, this bilayer apparently contained at least seven active channels. (*B*) Conductance is 77 pS and the reversal potential is −23.8 mV.

(Segal and Reuss, 1989), or by reconstitution into lipid bilayers (Reinhardt et al., 1987; Landry, Akabas, Redhead, Edelman, Cragoe, and Al-Awqati, 1989; Reeves and Andreoli, 1990). However, no such channels have been isolated in pure form, the detailed molecular mechanisms underlying their action remain unclear, and the measured single channel conductances vary over a wide range. Recently, epithelial chloride channels have been of particular interest because of their importance in two major clinical conditions, namely cystic fibrosis, a disorder which affects chloride channels in a number of epithelial systems, including the airways (Knowles, Gatzy, and Boucher, 1983; Widdicombe, Welsh, and Finkbeiner, 1985; Boucher, Stutts,

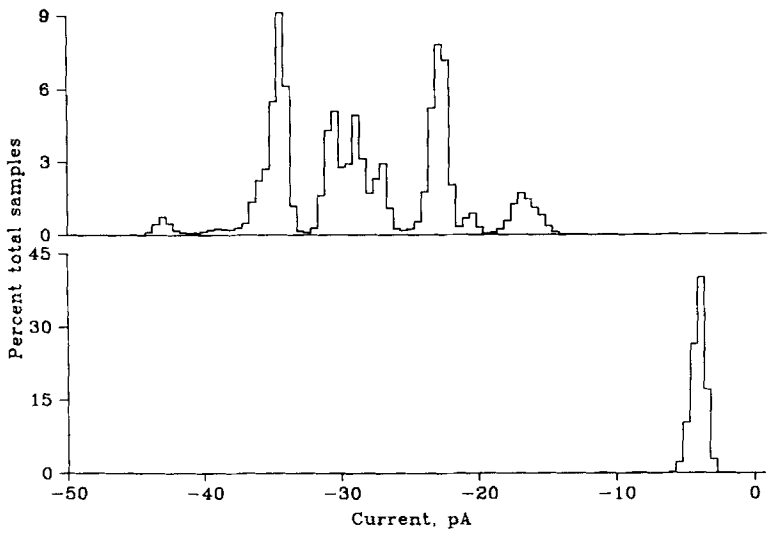

FIGURE 13. Amplitude histogram, −60 mV (channel openings at this potential are negative). These data were obtained from the channels shown in Fig. 10 and include all data at that potential (176 s before antibody, and 77 s beginning 30 s after antibody was added). The data on the bottom show a complete disappearance of all current levels seen in control traces, and the appearance of a distribution of current levels indicating the presence of few states or substates, at much lower amplitudes than in the control condition. The major amplitude shown (4.3 pA) is above the bare bilayer level, and is the current expected for a single open channel. There are barely detectable levels at about −8.5 and −11 pA.

Knowles, Cantley, and Gatzy, 1986; Frizzell et al., 1986; Welsh and Liedtke, 1986), the pancreas (Quinton, 1983), the sweat gland (Sato and Sato, 1984), and the large (Goldstein, Nash, Al-Bazzaz, Layden, and Rao, 1988) and small (Berschneider, Knowles, Azizkhan, Boucher, Tobey, Orlando, and Powell, 1988; Taylor, Baxter, Hardcastle, and Hardcastle, 1988) intestine, and secretory diarrheal diseases, which may also be due to changes in the activity of chloride channels (Guerrant, 1985; Powell, 1987).

Our approach has been to study NGB as a model epithelium that contains chloride channels and whose mechanisms of solute and water transport are similar to those of

the intestine. In each of the other epithelial chloride channels so far described, including, for example, the trachea (Shoemaker et al., 1986), the rectal gland (Greger and Schlatter, 1984), and cultured A6 epithelial cells (Nelson et al., 1984), the conductance pathway is stimulated by cAMP, shown either by direct addition of the compound or one of its analogues, by forskolin which raises the intracellular level of cAMP, or by inhibition of phosphodiesterase. In NGB, a large apical chloride conductance is induced by cAMP (Petersen and Reuss, 1983) or by forskolin (Poler and Reuss, 1987). In fact, this tissue is the only tissue in which it has been shown that a chloride conductance can be activated by an appropriate stimulus, but in which no

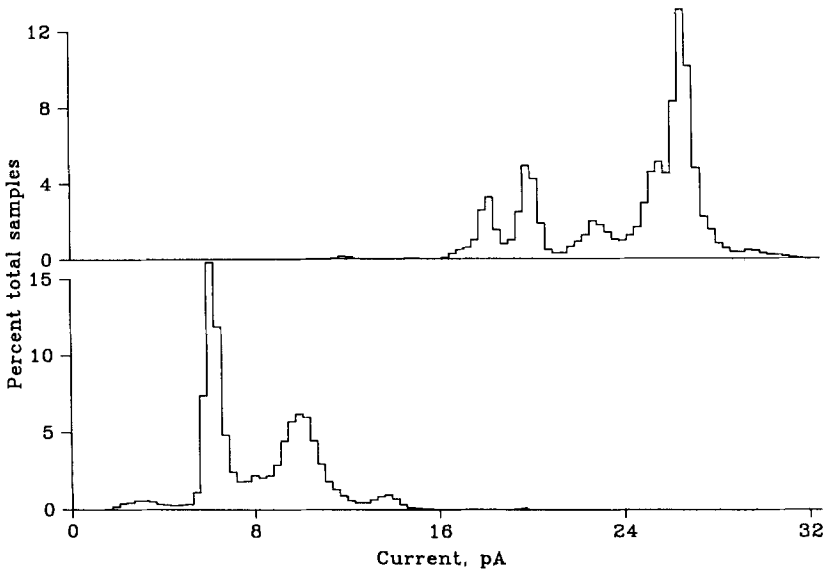

FIGURE 14. Amplitude histogram, 0 mV (at this potential, channel openings are positive) from a different experiment. The bilayer potential was held at 0 before and after the addition of antibody, and we were able to record at this potential for many minutes. All data at 0 mV are shown, and include 237 s of recording before antibody, and 650 s after. The overall effect is a dramatic shift to lower-current states, with minimal overlap with the control distribution. Amplitude distributions obtained as a function of time at 1–2-min intervals after antibody addition showed that there were no significant changes after the first 30 s (not shown).

such conductance can be detected under control conditions. The apparent magnitude of the pathway (30–40 mS/cm^2) (Poler and Reuss, 1987) makes this channel an attractive candidate for isolation, purification, and characterization by the methods of molecular biology and biochemistry.

In this study, using a monoclonal antibody that blocks the apical chloride conductance in NGB epithelium, we examined its cross-reactivity with several of the tissues mentioned above, which are known to contain chloride channels. The results of immunoabsorption and ELISA studies indicate that MAb E12 cross-reacts with different tissues from various species; reactivity is significantly diminished after the

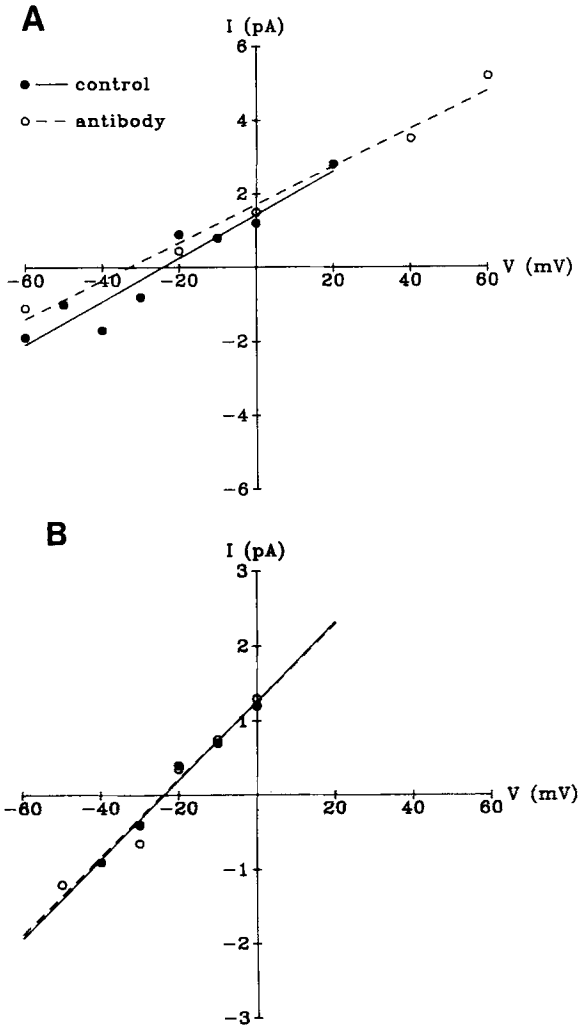

FIGURE 15. Effect of antibody on apparent unitary currents. In each case, current–voltage data obtained before antibody are shown as solid lines and filled circles, and those obtained after antibody are shown as dotted lines and open circles. The data were obtained from unitary transitions seen at each voltage, from traces similar to those shown in Fig. 12. (*A*) Data from the experiment shown in Figs. 10 and 13. Single channel conductance before antibody is 58.8 pS, and reversal potential is −24.4 mV; after antibody the values are 51.7 and −33.1, respectively. (*B*) Data from the experiment of Fig. 14. Single channel conductance before antibody is 53.0 pS and reversal potential is −23.8 mV; after antibody the values are 52.1 and −24.1, respectively.

antibody is absorbed with several different antigens, strongly supporting the hypothesis that these different cell sources share common epitopes. In addition, Western blot analysis demonstrates the recognition of a protein band of M_r 219,000 by MAb E12 in a variety of chloride channel-containing tissues spanning several species from amphibians to human, indicating a wide phylogenetic conservation of this protein.

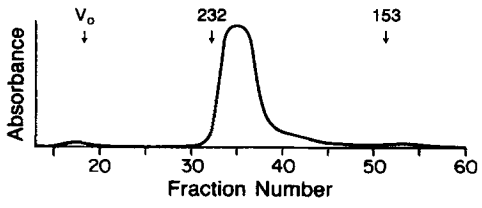

FIGURE 16. Gel filtration analysis. Absorbance at 254 nm is plotted in arbitrary units on the ordinate, against fraction number on the abscissa. V_0 is the void volume. Molecular weight markers (*arrows*) were catalase (232 kD) and aldolase (153 kD).

Surprisingly, the antibody also reacts by ELISA with an extract of *Torpedo californica* electric organ. In view of the evidence presented here that this antibody blocks a reconstituted chloride channel, this observation raises the possibility that the epithelial chloride channel may have homology even with tissues apparently quite unrelated. This is of special interest, of course, since the chloride channel from *Torpedo marmorata* was recently cloned and expressed in *Xenopus* oocytes (Jentsch, Steinmeyer, and Schwarz, 1990); the primary structure of this voltage-gated, outwardly rectifying channel indicates a molecular mass of 89 kD. At this time it is not possible to say what the relationship is between that channel and the one described in this report.

Although no other antibody has been shown to have more than a minimal inhibitory effect on an ion channel, our electrophysiological data show that MAb E12 blocks chloride channel activity in *Necturus* and toad gallbladder, rat colon, and human nasal epithelium. Furthermore, the antibody is highly specific in its action on the chloride channel; that is, it has no effect on resting transepithelial membrane potential or resistance, fractional apical resistance, or apical membrane conductances to sodium or potassium in NGB. However, our evidence that the antibody inhibits the chloride conductance in various epithelial tissues does not speak to the mechanism of the inhibition. It could be that the antibody binds not to the channel itself but to a regulatory molecule that either controls a channel gating mechanism or affects channel insertion or removal from the membrane. The latter possibility is less likely since we have previously shown that the antibody binds to NGB even in the absence of a functioning chloride conductance and, in that circumstance, prevents subsequent opening of the channel by theophylline-induced elevation of cellular cAMP (Finn et al., 1989). Thus the channel molecule is likely to be present in the apical membrane at rest, and an effect of the antibody on a fusion or endocytotic process is unlikely. Furthermore, the reconstitution experiments strongly suggest a direct action of some kind between antibody and channel; that is, these experiments seem to have ruled out an interaction of antibody with a regulatory molecule unless the latter is covalently linked to the channel protein.

The reconstitution experiments were done using a simple but well-described method, as noted above. We chose this method initially because it was relatively simple and did not require initial reconstitution of the channel protein into liposomes. Since the method has worked regularly (58 of 66 times, as already stated), we have continued to use it. As noted in the Results, control experiments (using detergent alone, myeloperoxidase with detergent, or gallbladder protein preparations heated in boiling water for 5 min) did not yield channels, strongly supporting our conclusion that the observed channels represent reconstitution of the purified gallbladder protein.

The antibody seems to affect the reconstituted channels much more rapidly (30 s or less) than the in situ chloride conductance (10 min seems to be the minimum time required [Finn et al., 1989]). There are many possible reasons for this discrepancy, none of which can be assessed accurately at the present time. The most obvious are that access to the binding site may be much greater in the planar bilayer for a number of reasons, that we do not stir the antibody when it is placed on the tissue, and that the sensitivity of the measurement of channel activity in the bilayer

experiments is orders of magnitude greater than the measurement of chloride conductance in situ.

In any case, we have been able to reconstitute a channel that is highly selective for chloride over sodium, and is similar to some chloride channels previously reconstituted. In several cases, multiple conductance states have been clearly seen either in patches from cell membranes (Geletyuk and Kazachenko, 1985; Krouse, Schneider, and Gage, 1986) or in channels reconstituted into planar bilayers (Meves and Nagy, 1989). Although a wide variety of chloride channels has been described, many of them have conductances quite similar to the ones we have reconstituted; these include, for example, channels obtained from human respiratory cells (Schoumacher, Shoemaker, Halm, Tallant, Wallace, and Frizzell, 1987; Li, McCann, Liedtke, Nairn, Greengard, and Welsh, 1988; Jetten, Yankaskas, Stutts, Willumsen, and Boucher, 1989), rabbit kidney (Reeves and Andreoli, 1990), shark rectal gland (Greger, Schlatter, and Gogelein, 1987), and rat colon (Reinhardt et al., 1987). Interestingly, antibody E12 blocks chloride conductance in both human respiratory cells and rat colon, and partly blocks the chloride channels reconstituted from rabbit kidney cells (Reeves, W. B., and T. Andreoli, personal communication). Thus it would seem that this antibody recognizes and can alter the function of a wide variety of well-characterized chloride conductance pathways.

From the behavior of the channel under both control and antibody-treated conditions we can gain some insight into its characteristics. It has a conductance in 150/50 mM NaCl of ~60 pS, although it may exist in various subconductance states as well. We believe that other levels represent multiples of a single open channel with the same unit conductance, and that the large transitions represent the simultaneous gating of several such channels. We cannot derive a kinetic scheme or determine the voltage dependence of the gating because of the multiplicity of channels and our inability up to now to incorporate only one or two of them. In most instances there were multiple open states from the very first increase in bilayer conductance, suggesting that the channels behave as aggregates or that they open in a cooperative manner, rather than as consecutive incorporations of independent channels. This conclusion is strengthened by the data in Fig. 10, which show frequent transitions (both opening and closing events) spanning multiples of the 60-pS level, a statistical impossibility if all of the levels represented multiple gating events of single independent 60-pS channels.

One way that these data can be explained is if the channels can exist in the membrane both as individual units and as aggregates, such that the aggregated units open and close simultaneously without affecting the gating of nonaggregated individual units (Geletyuk and Kazachenko, 1985; Krouse et al., 1986). This hypothesis is consistent with the observation that the apparent unitary events were independent of the large transitions and that both single and aggregated channels could exist in any given bilayer. In this case, the effect of antibody would be to inhibit the gating of the aggregates, favoring a decreased open probability without changing the conductance of the individual units.

Alternatively, it is possible that the channels exist only as aggregates of individual units, with multiple or cooperative gating mechanisms. In this case, the large-conductance transitions would be due to a rapid cooperative gating of individual

units. The observation of individual unitary events suggests that the cooperativity of the gating was not always complete, allowing some individual transitions to occur from any given conductance level without triggering a large transition. In this gating scheme, the effect of antibody would be to lower the probability of cooperative gating without affecting the unitary events.

As noted in Figs. 10 *B*, 13, and 14, the effect of antibody is to shift the open states to drastically lower levels, but there is no effect on the current–voltage characteristics of the smallest detected, or unitary, transitions (Fig. 15). It is clear from these data that the large transitions are almost completely lost (for example, after the beginning of the second trace in Fig. 10 *B*), but that the antibody does not reduce the current level to 0. The amplitude histograms also make this clear, and the drastic reduction in amplitudes never comes about by sequential closing of unitary channels, but only by the loss of the large transitions. Taken together, these results suggest that the channels are aggregated in some way, that the aggregate is gated independently of the single channels, and that it is the large gate (i.e., the one that controls the aggregate) that is affected by the antibody. These large transitions cannot be independent maxi-channels for the reasons already mentioned, and because observation of all the records indicates that there are transitions of many different sizes, from a minimum observable transition (which we are calling the unitary, or single channel, transition) to a maximum which is several (sometimes as much as 10) times that size; there is no common large transition that can be designated a maxi-channel, and it is therefore far more likely that we are seeing aggregate behavior, as already described. Examples of this behavior are seen in most of our records; one such case is shown in Fig. 11, taken from the same study as shown in Fig. 10, in this case with the transmembrane potential clamped at 0 mV. As described in the legend, this 4-s period demonstrates transitions of almost all levels observed in the entire experiment. That the multi-level transitions are also chloride channels is clear from the observation that if we construct a current–voltage curve from the largest transitions observed at each voltage (instead of from the unitary events shown in Figs. 12 and 15), we find a reversal potential identical to that obtained from the unitary transitions (-25.3 ± 0.7 mV; data were obtained from the same 11 incorporations mentioned in Results).

Further experiments are needed to sort these characteristics out; one way to do this may be to insert the proteins into vesicles and then fuse them into the bilayer, a technique that has been frequently used when dealing with pure or semipure proteins (Cohen, Zimmerberg, and Finkelstein, 1980; Miller, 1982). We are presently testing anion selectivity, the effects of inhibitors, the concentration dependence of antibody interaction, and a variety of other subjects related to this channel.

The reconstituted channels are open almost all of the time, unlike the resting channel in the native tissue which was the source of the antibody, NGB. This is not entirely surprising, since the environment of the channel in situ is surely different from that in the bilayer. Evidence that we have reconstituted the same channel as that which exists in vivo is strong: the antibody blocks only the chloride conductance in the apical membrane of the native tissue, it recognizes a band of the same molecular weight in both crude and immunopurified material, and the antibody blocks the channel in the bilayer.

Our data indicate that the channel has a molecular mass of ~219,000 D, but verification awaits purification and sequencing. Although it is impossible to know the molecular weight of the specific species incorporated into a planar lipid bilayer, the fact that FPLC-fractionated material of approximately the same molecular weight yielded an identical channel in the bilayer supports our conclusion. Our electrophysiological data, along with the Western analyses, strongly suggest that the protein recognized by antibody E12 is the apical chloride channel, or a component of it, and that such a molecule is strikingly conserved over much of the animal kingdom.

In conclusion, we have used a monoclonal antibody that inhibits chloride conductance in a wide range of epithelial tissues as the ligand in an immunoaffinity column, and have succeeded in purifying a protein of M_r 219,000; when this purified material is reconstituted into a planar lipid bilayer, it behaves as a chloride-selective channel which is blocked by the antibody.

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