Monoclonal antibodies to the apical chloride channel in *Necturus* gallbladder inhibit the chloride conductance

(Ion channels/membrane transport/epithelia)

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ABSTRACT  Monoclonal antibodies raised by injecting *Necturus* gallbladder cells into mice were tested for their ability to inhibit the apical chloride conductance induced by elevation of cellular cAMP. Five of these monoclonal antibodies bound to the apical cells, as shown by indirect immunofluorescence microscopy, and inhibited the chloride conductance; one antibody that bound only to subepithelial smooth muscle, by indirect immunofluorescence microscopy, showed no inhibition of chloride transport. The channel or a closely related molecule is present in the membrane whether or not the pathway is open, since, in addition to inhibiting the conductance of the open channel, the antibody also bound to the membrane in the resting state and prevented subsequent opening of the channel. The antibody was shown to recognize, by ELISA, epitopes from the *Necturus* gallbladder and small intestine. Finally, by Western blot analysis of *Necturus* gallbladder homogenates, the antibody was shown to recognize two protein bands of *M*₂ 219,000 and *M*₁ 69,000. This antibody should permit isolation and characterization of this important ion channel.

The *Necturus* gallbladder transports salt and water isotonically from the mucosal to the serosal solution; the first step in this transport pathway is the entry of NaCl at the apical border by way of the coupling of neutral Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers (1). Apparently independent of the salt and water transport are the conductive pathways in that membrane; they include a dominant potassium conductance and a small sodium conductance but no chloride conductance under control conditions (2). It has been shown (3, 4), however, that elevation of intracellular cAMP leads to the appearance of a chloride conductance large enough to dominate the apical membrane, the overall increase in membrane conductance being 200-300-fold. Because epithelial chloride channels play a critical role in secretion of this ion (5–7) and because of the apparent abundance of such channels in the stimulated *Necturus* gallbladder, we decided to use this tissue as a model to characterize the channel. We now report that we have raised monoclonal antibodies that recognize protein bands of *M*₂ 219,000 and *M*₁ 69,000 and that almost completely block the conductance of the channel. Furthermore, we show that when the channel is a constituent of the apical membrane under control conditions and that the action of the presumed cellular messenger (cAMP) is to activate the channel rather than to cause its insertion into the membrane.

METHODS

Preparation of Monoclonal Antibodies. Epithelial cells were scraped from gallbladders exposed to 5 mM theophylline for 5–15 min and were subjected to nitrogen cavitation at 400 psi (1 psi = 6.9 kPa) (8); the resultant supernatant was used as the immunogen. The first injection consisted of this immunogen prepared in complete Freund’s adjuvant (to enhance immunogenicity), whereas subsequent antigen administrations were diluted in isotonic phosphate-buffered saline (PBS). Serum obtained from one of the immunized (BALB/c) mice demonstrated enhanced immunostaining of the apical membranes of gallbladder cells by indirect immunofluorescence and totally inhibited the theophylline-induced chloride conductance in a single preparation, whereas serum that showed no immunostaining (from one of the other injected mice) had no effect on the chloride conductance. Hybridomas were then prepared using standard techniques (9). Briefly, 4 days after the final immunization, spleen cells obtained from the mice were placed in Dulbecco’s modified Eagle’s medium and fused with cells from the BALB/c cell line P3-NS1/1-AG4-1 in 50% (wt/vol) polyethylene glycol (PEG-1000; J. T. Baker). The cells were grown in 96-well microtiter plates (Costar) in HAT medium containing hypoxanthine, aminopterine, and thymidine. Clones were screened by indirect immunofluorescence microscopy for monoclonal antibodies that stained gallbladder cells. Positive clonal supernatants were then tested for their ability to inhibit chloride conductance, and hybridomas secreting monoclonal antibodies were cloned by limiting dilution.

For immunofluorescence microscopy, frozen sections of *Necturus* gallbladder were exposed to each of the monoclonal antibodies tested, washed free of antibody, then exposed to fluorescein isothiocyanate-labeled heavy- and light-chain-specific goat anti-mouse IgG, and viewed and photographed through a fluorescence microscope.

ELISA was carried out on poly(L-lysine)-coated Costar microtiter plates. Various concentrations of epithelial cell homogenates from *Necturus* gallbladders or small intestines were added for 1 hr at room temperature to the wells and then incubated overnight at 4°C. This procedure was followed by washing three times with PBS, incubating with 1% gelatin for 1 hr, and then washing three more times. Supernatant from hybridoma clones or medium alone was added and incubated for 1 hr at room temperature. After three washings with PBS, the plates were incubated for 30 min at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Tago). The substrate for the peroxidase reaction was a solution of 0.04% o-phenylenediamine dihydrochloride in 0.15 M sodium citrate/0.15 M sodium phosphate, pH 5.0, with 0.012% hydrogen peroxide. Optical density was read after 30 min at 450 nm with a Microplate reader (Bio-Tek Instruments, Windoski, VT).

Preparation of the *Necturus* gallbladder for polyacrylamide gel electrophoresis was performed using several procedures. Ultimately, the most reliable technique proved to be sonication in the presence of protease inhibitors. The polyacrylamide gels were electrophoresed under denaturing conditions with

Abbreviation: Clᵢ, chloride activity of the cell.

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and without the reducing agent, 10% (vol/vol) 2-mercaptoethanol. The most useful gels were those made of 5% or 5–10% polyacrylamide gradients. Proteins were transferred electrophoretically from gels to nitrocellulose for Western blot analysis; transferred protein was visualized on portions of the nitrocellulose by staining with Amido black. Individual lanes were blocked with 0.5% Tween 20, washed, then incubated overnight with primary antibody. They were subsequently washed and incubated with peroxidase-conjugated goat antimouse IgG and IgM.

**Electrophysiological Studies.** Gallbladders were removed from pithed animals and placed, mucosal side up, across a 0.5-cm² opening between two Lucite chambers. Both sides of the tissue were perfused with a solution containing 95 mM NaCl, 10.0 mM NaHCO₃, 1.2 mM K₂HPO₄, 0.1 mM KH₂PO₄, 1.0 mM MgCl₂, and 2.0 mM CaCl₂ that was gassed with 99% O₂/1% CO₂ and had a pH value of ~7.9. Transepithelial potential was measured with silver/silver chloride electrodes inserted into glass capillaries such that the electrodes made contact with the medium through a small length of normal bathing medium. Transepithelial current was passed through platinum/iridium electrodes in each bathing medium.

Double-barreled electrodes (10, 11) were prepared from fused glass capillaries (1-mm o.d., 0.5-mm i.d.) with inner filaments (Hilgenberg, Masfeld, F.R.G.) that had been pulled and twisted to make microelectrodes with tip diameters <0.5 μm. The tips of the ion-selective barrels were backfilled with Cl⁻ liquid ion-exchange resin (number 477913, Corning) after they had been silanized with hexamethyldisilazane (Fluka). The resins were allowed to fill the tips by capillary action at room temperature (21–22°C) and the rest of the barrel and the reference barrel were filled with 0.5 M KCl. Microelectrodes were connected to the probes of a two-channel high-impedance electrometer (model FD 223; W-P Instruments, New Haven, CT) by a chlorided silver wire sealed into the shank of each barrel; the output from the reference barrel was electronically subtracted from that of the ion-selective barrel, and this difference (proportional to the ion activity) was displayed, along with the voltage of the reference barrel, on an oscilloscope and recorded by a computer.

The electrodes were calibrated at room temperature in pure single-salt solutions and in media similar to those to be used in an experiment. The slope of the recorded potential difference against ion activity in the solution was determined over a range of 10–100 mM Cl⁻ (the slopes range from 50–54 mV per decade) and intracellular ion activities were calculated from the Nicosky equation.

An example of the measurement of chloride conductance is shown in Fig. 1. Under control conditions (Fig. 1A), the reduction of mucosal Cl⁻ from 101 mM to 6 mM (by cyclamate replacement) led to cell hyperpolarization. The initial sharp change of about 7 mV was due to the liquid junction potential (determined by measuring the changes in potential when a KCl/agar bridge was substituted for the gallbladder preparation and the same unilateral solution changes were made) and the subsequent slower phase in part to the cellular alkalization (with attendant rise in potassium conductance) that results from decreased apical Cl⁻/HCO₃⁻ exchange (12).

The voltage deflections hardly changed, indicating no change in the relative resistance of the apical membrane (there was no significant change in transepithelial resistance in this experiment or in the one shown in Fig. 1B, so that the change in the deflection across the apical membrane is a measure of the relative resistance of that membrane), and cell chloride activity (Clᵦ) decreased monotonically from an initial value of 26.3 mM (well above the calculated equilibrium value of 6.2 mM) to 11.7 mM by about 50 sec after the change in medium Cl⁻ and remained constant thereafter. Thus there was no evidence for an apical chloride conductance. In Fig. 1B, 0.5 mM theophylline had been added to the serosal side, the

![FIG. 1. Data are from two cells in the same tissue; the pulses represent the response of the membrane potential to the passage of brief pulses of current and are a measure of the relative resistance of the apical membrane. Upper traces are the apical membrane potential Vₘₑₙₐ and lower traces are the difference between the ion-selective and reference electrode; the ordinate is calibrated in terms of chloride activity in mM. (A) Control period. (B) Same tissue (different cell), 5 min after the addition of 0.5 mM theophylline to the serosal side.](image-url)

resting membrane potential has considerably depolarized, and the relative apical resistance was much lower than in the control cell in Fig. 1A.

Now a similar reduction in medium Cl⁻ led to a marked depolarization and a doubling of the relative resistance of the apical membrane (as indicated by the increase in the voltage deflections from the transepithelial pulses). At the same time, Clᵦ decreased from 14.2 mM (equal to the equilibrium value at this membrane potential) to 4.2 mM, at a much more rapid rate than occurred in the absence of theophylline. The change in potential was transient, and the repolarization was due to the rapid loss of cell chloride and the resulting increased effect of the potassium conductance on the membrane potential. These changes were readily reversible after the removal of theophylline, and all were similar to those previously described (3, 4). They can be attributed to the increase in cellular cAMP that follows inhibition of phosphodiesterase by theophylline, since (i) this compound reproducibly elevates cAMP levels in other epithelia (13, 14) and (ii) the same changes occur when either 8-bromoadenosine 3',5'-cyclic monophosphate or forskolin (refs. 3 and 4; A.L.F., unpublished data) is added to the serosal medium.

We found that the magnitude of the change in potential for a given change in chloride concentration was dependent on the concentration of theophylline over a range of 0.1–1.0 mM and was reproducible for any given tissue even after repeated removal and readdition of the drug; we routinely titrated the latter to find a concentration that would yield a submaximal response. We next removed the mucosal solution, placed a monoclonal antibody solution on the mucosal surface of the cells at room temperature for 15 min, and then removed unbound antibody. We then repeated the change in medium chloride and determined the magnitude of the early depolar-
ization, as described above. We used the magnitude of the depolarization as a semiquantitative estimate of the conductance of the apical membrane to chloride. In some experiments we reversed the direction of the chloride changes. That is, the tissues were initially perfused on both sides with the 6 mM (low) chloride solution, so that all tissues would presumably have a low and, therefore hopefully, more consistent chloride concentration in all conditions; they were then tested in the presence or absence of theophylline or antibody by raising the medium Cl\(^-\) concentration to 106 mM. The results were essentially unchanged, the potential, resistance, and Cl\(^-\) changes simply being the mirror image of those shown in the figures. Controls were of two kinds: (i) Repeated chloride reductions (in multiple cells in a given tissue) during continual perfusion with theophylline indicated clearly that there was no decrease in chloride conductance (as measured by the Cl\(^-\)-dependent potential change) for at least 5 hr. (ii) Incubation of the cells with a monoclonal antibody that recognized only smooth muscle by immunofluorescence had no effect on the conductance (see below).

In all tissues, the responses to changes in medium chloride were tested in at least five cells in the presence (as in Fig. 1A) and absence (Fig. 1B) of theophylline prior to the addition of antibody, as described above.

RESULTS

Five of the monoclonal antibodies were found to inhibit chloride transport significantly, although the degree of inhibition varied. The most inhibitory reduced the chloride-dependent potential by 83%, from 16.6 ± 2.3 mV to 2.8 ± 1.3 mV (four bladders, 24 cells before antibody, and 28 cells after antibody; \(P < 0.001\)). The other four monoclonal antibodies reduced the response by 41–72%. Each of these antibodies showed binding to the epithelial cells by indirect immunofluorescence microscopy (Fig. 2 Left). On the other hand, one of them bound only to subepithelial smooth muscle (Fig. 2 Right), and this antibody had no effect on the theophylline-induced chloride conductance: the response to chloride change before incubation with the antibody was 17.3 ± 2.4 mV and that after incubation was 16.5 ± 3.5 mV (three bladders, 15 impaled cells before antibody and 18 impaled cells after antibody). The supernatant from another clone that did not recognize gallbladder cells by immunofluorescence microscopy showed neither binding nor inhibition of chloride conductance. Subsequent studies reported in this manuscript involve only the most inhibitory of the monoclonal antibodies.

Because of the possibility that the increase in cAMP induced by theophylline leads to the addition of new channels to the membrane, rather than simply causing the activation of constitutive channels, we exposed the tissues to antibody in the absence of theophylline. The experiments were performed as follows. Cells were impaled and chloride changes were made in the absence of theophylline to ascertain that the chloride channels were closed. Theophylline was then added, and cells were impaled and the response of the tissue to a decrease in mucosal chloride concentration was determined. The theophylline was removed, other cells were impaled, solutions were changed, and we ascertained that the channels were again closed. At this point, either the specific or control (to smooth muscle) antibody was added and incubated as described above. The excess antibody was removed by washing, theophylline was added, and the chloride-dependent membrane potential was determined. In four tissues to which the inhibitory antibody was added, the channels could not be opened by the same dose of theophylline that had opened them previously. The mean chloride-dependent potential change before antibody incubation was 16.0 ± 1.6 mV and that after antibody was 3.1 ± 1.0 mV \((P < 0.001)\). On the other hand, when the smooth-muscle antibody was added to four tissues, the response after antibody, 18.1 ± 1.6 mV, was not significantly different from that before the incubation, 16.4 ± 3.7 mV.

The reactivity of the monoclonal antibody with Necturus gallbladder was tested by both ELISA and then more specifically by polyacrylamide gel electrophoresis with Western blot analysis.

Fig. 3 shows the results of ELISAs performed with this monoclonal antibody; we have virtually identical results on two others. As shown, the same antibody that is directed against Necturus gallbladder (and indeed was obtained by immunizing the animal with Necturus gallbladder cells) also reacted with the intestinal antigen with almost the same avidity. The native gallbladder epithelial cells gave a satisfac-

**Fig. 2.** Indirect immunofluorescence microscopy. (Left) Antibody-binding pattern observed with all of the inhibitory antibodies; although this tissue had not been treated with theophylline, other studies showed that there was no difference in the staining characteristics whether or not the drug was present. There is heavy staining of the epithelial cells, with apparent accentuation at the apical border. Staining of the subepithelial tissue is indistinguishable from background staining seen with the fluorochrome alone. (Right) Section stained with one of the two noninhibiting antibodies. Fluorescent staining is completely localized to smooth muscle; antibody binding to the cells atop the smooth muscle and lined up adjacent to the cells on the left micrograph was not observed.

**Fig. 3.** ELISA. The optical density at 450 nm of the reaction product of horseradish peroxidase is plotted against the inverse concentration of antigen—that is, of cell protein obtained by freezing and thawing. The scale is such that the leftmost point on the graph represents a concentration of 7.5 \(\mu\)g of antigen per well, and the rightmost point is 0.1 \(\mu\)g of antigen per well. Closed circles, Necturus gallbladder epithelial cells as the source of the antigen; open circles, small intestinal cells from the same animal.
Theory response that was reasonably sensitive, since 100 ng gave a reading that was more than twice the background level. This assay was done on a treated preparation, that is, one that had been exposed to theophylline before the cells were removed for harvesting the protein. Using the same protein concentration, non-stimulated cells yielded the same results, suggesting that the presence of open channels on the apical membrane is not necessary for recognition by the antibody.

Western blots of denatured proteins revealed immunoreactivity of the inhibitory monoclonal antibody with proteins of Mr 219,000 and Mr 69,000, as shown in Fig. 4, lane A. When these proteins were electrophoresed under reducing conditions (Fig. 4, lane B), it appears that two bands were found at Mr 219,000 and Mr 213,000 and another two bands were at Mr 69,000 and Mr 63,000, suggesting that perhaps the native proteins are dimeric and split under reducing conditions.

**DISCUSSION**

Chloride channels, or conductance pathways, exist in a number of epithelia; it is now clear that our understanding of at least two disease processes will be improved by characterizing these pathways in the intestine and similar tissues. Those disease processes are (i) the inherited disease cystic fibrosis, which affects chloride channels in the airways (15), the pancreas (16), the sweat gland (17), and the large (18) and small (19) intestines, and (ii) acquired secretory diarrheal diseases, which may also be due to changes in the activity of such channels (20). The gallbladder, like the small intestine, contains similar chloride channels and, at least in several mammalian species, is capable of carrying out net water and electrolyte secretion, much like the intestine, during hormonal (21) and drug (22) treatment and in inflammation (23). The gallbladder, therefore, is a reasonable model of the small intestine and has the great advantage of being a simpler epithelium containing, at least in *Necturus* and other amphibians, only a single cell type.

As part of our approach to the study of such channels, we present data here on our preparation of monoclonal antibodies that bind to *Necturus* gallbladder cells and inhibit the cAMP-induced chloride conductance pathway in the apical membrane of those cells. As discussed above, each of the five antibodies showed identical patterns by immunofluorescence microscopy, which indicated (Fig. 2) predominant binding at the apical membrane. However, as is also shown in Fig. 2, it is clear that the antibody recognizes sites within the cells as well. This may indicate the presence of similar antigens on intracellular membranes or organelles.

The most inhibitory of these antibodies almost completely blocks the chloride conductance in the tissue from which it was derived. It recognizes two major bands by immunoblot, at Mr 219,000 and Mr 69,000. Of interest is that one or both of these proteins, possibly the chloride channel itself or a closely related molecule, is also recognized by the antibody when the channel is closed (i.e., inactive), indicating that it is constitutive and that the role of cAMP is to control its activation rather than its insertion into the membrane. The antibody should prove to be an extremely useful ligand for the isolation and characterization of this important channel.

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