Colistin interactions with the mammalian urothelium

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Submitted 9 October 2003; accepted in final form 2 December 2003

Lewis, Jamie R., and Simon A. Lewis. Colistin interactions with the mammalian urothelium. Am J Physiol Cell Physiol 286: C913–C922, 2004.—Here we describe the effect of colistin on the barrier function of the mammalian urinary bladder epithelium. Addition of colistin to the mucosal solution of the rabbit urinary bladder epithelium (urothelium) resulted in an increase in the transepithelial conductance. The magnitude of the increase in transepithelial conductance was dependent on the membrane voltage, concentration of colistin, and presence of divalent cations in the bath solution. The initial site of action of colistin was at the apical membrane. Colistin increased the membrane conductance only when the apical membrane potential was cell interior negative. The more negative the membrane potential, the larger the conductance increase. The concentration dependence of the conductance increase saturated, suggesting a membrane binding site. Divalent cations decreased the magnitude of the conductance increase. This divalent cation action occurred at two sites: one in competition with colistin for a membrane binding site, and the other by rapidly blocking the induced conductance. At short exposure times, the increase in conductance was reversed by either removing colistin from the bath or changing the voltage so that the apical membrane was cell interior positive. At long exposure times, the increase was only partially reversible by voltage or removal from the bath. This finding suggests that at long exposure times, there is a toxic effect of colistin on the urothelium.

Colistin (also known as polymyxin E) is an antibiotic that was isolated in 1950 from Bacillus colistinus. Because of its nephrotoxicity and neurotoxicity, use of colistin is restricted to isolated in 1950 from Bacillus colistinus. Although the amino lipid soluble. Of interest is that colistin is different from make colistin amphipathic in nature, i.e., readily water and diaminobutyric acid. The cationic nature and the fatty acid tail of P. aeruginosa, with PMB being more potent. Similarly, Nord and Hoeprich (10) compared the bactericidal activity and toxicity to mice of the two drugs and found PMB to be the more potent of the two drugs; however, for equivalent bactericidal activity, colistin was as toxic as PMB (10).

The bactericidal activity of polymyxin is caused by its ability to increase the permeability of the bacterial membrane. Three key features for bactericidal activity are the fatty acid tail, positively charged amino acids, and the peptide ring (see Ref. 17 for review). Loss of any one of these features results in loss of bactericidal activity. Bactericidal activity can be decreased by altering fatty acid chain length (see Ref. 12 for a review), decreasing the number of charged residues, altering the length of the ring, altering the length of the charged side chain, and altering the amino acid composition of the hydrophobic domain (14).

Previous studies (1, 2) have demonstrated that PMB is toxic to the urinary bladder epithelium. The proposed mechanism of PMB toxicity is by increasing membrane permeability, resulting in an increased influx of cations, anions, and water, leading to cell swelling and lysis. These authors performed a detailed analysis of the interaction of PMB with the urinary bladder membrane. The purpose of this study is to compare and contrast the effect of colistin to PMB on the mammalian urinary bladder epithelium, with the goal of determining whether colistin is less toxic to the bladder than PMB and the basis for any difference in toxicity.

MATERIALS AND METHODS

Tissue Preparation

The animal experiments were performed in accordance with the University of Texas Medical Branch animal care and use committee. Male New Zealand White rabbits (3 kg) were euthanized by lethal intravenous injection of sodium pentobarbital. Urinary bladders were excised and washed in a NaCl Ringer solution (see Solutions). The smooth muscle was dissected away, and the epithelium was mounted on a ring of 2-cm² exposed area. The ring was transferred to a temperature-controlled, modified Ussing chamber (7) where the serosal side of the epithelium was held against a nylon mesh by a slight excess of solution in the mucosal chamber. Both the mucosal and serosal chambers were stirred by magnetic spin bars at the bottom of each chamber.

Solutions

NaCl Ringer contained (in mM) 111.2 NaCl, 25 NaHCO₃, 10 glucose, 5.8 KCl, 2.0 CaCl₂, 1.2 KH₂PO₄, and 1.2 MgSO₄. In KCl Ringer, all Na⁺ salts were substituted with the appropriate K⁺ salts.

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Unless otherwise noted, all experiments were performed by using KCl Ringer as the mucosal bathing solution. Colistin was prepared as a stock solution by dissolving colistin in distilled H2O. Colistin was added in microliter quantities to the mucosal solution. Colistin was purchased from Sigma Chemical (St. Louis, MO).

Data Analysis and Statistics

Data Analysis and Statistics

Electrical Measurements

All electrical measurements were made under voltage-clamp conditions unless otherwise noted. The transepithelial voltage ($V_t$) was measured with Ag-AgCl wires placed adjacent to either side of the epithelium (serosal solution ground) while current ($I$) was passed from Ag-AgCl electrodes placed in the rear of each chamber. Both sets of electrodes were connected to an automatic voltage clamp (Warner Instruments). The transepithelial resistance ($R_t$) and its inverse, the transepithelial conductance ($G_t$), were calculated, using Ohm’s Law, from the current required to clamp the epithelium 10 mV from the holding voltage.

Data Acquisition

Current and voltage outputs of the voltage clamp were connected to an analog-to-digital converter (Scientific Solutions) interfaced with a computer, which calculated values for resistance and short-circuit current ($I_{sc}$). $V_t$ and $I$ were continuously monitored on an oscilloscope. All data were printed out with the time of data acquisition and additionally stored on the hard disk.

Equivalent Circuit Analysis

The method of Yonath and Civan (19) was used to differentiate between an increase in the conductance of the cell membrane or the tight junctions. The $G_t$ ($\mu$S/cm$^2$) was plotted as a function of $I_{sc}$ ($\mu$A/cm$^2$) when $V_t = 0$ mV in the presence of added protein. This plot was then fit by the equation

$$G_t = (I_{sc}/E_c) + G_j$$

This plot is linear if the added protein changes only the cell resistance when the transepithelial voltage is clamped to 0 mV. The y-intercept of the line will be equal to the junctional conductance ($G_j$), and the slope will be the inverse of the cellular electromotive force, which is the sum of the apical and basolateral membrane equivalent batteries, or $E_c$.

Current-Voltage Relationship

The steady-state difference current-voltage (IV) relationship of the protein-induced conductance was calculated by using the method of Tzan et al. (15). This method involves measuring the transepithelial IV relationships in both the absence and presence of added protein; the difference between these two relationships is the voltage dependence of the current flowing through the protein-induced conductance. First, the tissue was voltage clamped to $V_t = 0$ mV, and the transepithelial current responses to computer-generated voltage pulses 30 ms in duration and of increasing magnitude and alternating polarity were measured. Next, the transepithelial potential was voltage clamped to $-70$ mV, protein was added to the mucosal solution and equilibrated for 5 min, and then the transepithelial potential was clamped to 0 mV. The conductance was allowed to reach a steady state before the IV relationship was again measured. The difference between the IV relationships in the presence and absence of added protein was then fit by the constant field equation to determine the relative ionic permeabilities of the protein-induced conductance.

Data Analysis and Statistics

Curve fitting was done on a personal computer by using NFIT (Island Products, Galveston, TX). Statistics were calculated by using INSTAT (GraphPad Software, San Diego, CA). Data are shown as means ± SE.

RESULTS

Effect of Colistin on $G_t$

It was previously demonstrated that PMB increases the $G_t$ of the rabbit urinary bladder epithelium in a voltage-dependent manner (1). PMB was without effect on $G_t$ when the apical membrane voltage was cell interior positive (by $-15$ mV); however, when the apical membrane voltage was made cell interior negative, there was a rapid increase in $G_t$. This voltage sensitivity had the advantage of allowing an accurate determination of the kinetics of the increase in membrane conductance. The standard experimental protocol used was as follows. $V_t$ was clamped to $-70$ mV (this yielded an apical membrane voltage of $15$ mV cell interior positive), and colistin was added to the apical membrane bathing solution from a concentrated stock solution. After a 5-min incubation (to allow for diffusion through the unstirred layer), $V_t$ was clamped to 0 mV (an apical voltage of $-55$ mV cell interior negative) and the time course of $G_t$ followed. Figure 1A shows that at a $V_t$ of $-70$ mV, colistin did not alter $G_t$. When $V_t$ was subsequently clamped to 0 mV, there was a time-dependent increase in $G_t$.

Time Course of $G_t$ Increase

The time course of $G_t$ in response to 40 $\mu$M colistin was fit by an equation that was the sum of an exponential, a line, and the baseline $G_t$

$$G_t(t) = G_{col}^{max}(1 - e^{-kt}) + G_{t}^{Col-slow} \times t + G_{baseline}$$

where $G_t(t)$ is transepithelial conductance as a function of time $t$ (s), $G_{col}^{max}$ ($\mu$S/cm$^2$) is the magnitude of the exponential component of the colistin-induced conductance, $k$ (s$^{-1}$) is the rate constant of the exponential component, $G_{t}^{Col-slow}$ ($\mu$S/cm$^2$-s$^{-1}$) is the slope of the linear component of the colistin-induced conductance, and $G_{baseline}$ is the baseline transepithelial conductance (during curve fitting, $G_{baseline}$ was held constant at the value measured immediately before $V_t$ was clamped to 0 mV). Thirty-two percent of the time courses were described by the sum of an exponential and a slow linear increase (0.4 $\mu$S/cm$^2$-s$^{-1}$), sixty-two percent by only an exponential, and the remaining six percent by only a linear increase (9.4 $\mu$S/cm$^2$-s$^{-1}$). Table 1 gives the values for the magnitude and rate constant of the exponentials and rate of conductance increase for the linear responses. At lower colistin concentrations ($\leq 40$ $\mu$M), 80% of the responses were exponentials and 20% were linear. The rate constant for the exponential was not concentration dependent (4 $\mu$M: 0.054 ± 0.006 s$^{-1}$, n = 4; 10 $\mu$M: 0.044 ± 0.011 s$^{-1}$, n = 3; 20 $\mu$M: 0.033 ± 0.008 s$^{-1}$, n = 4; and 40 $\mu$M: 0.10 ± 0.014 s$^{-1}$, n = 21). At higher colistin concentrations ($\geq 100$ $\mu$M), the shape of the conductance increase could not be determined because the transepithelial conductance rapidly approached that predicted for the basolateral membrane. Because of the variability in the shape of the time course and to minimize changes in membrane voltage, the conductance increase was quantified by measuring the initial linear rate of conductance increase (see straight line in Fig. 1A).
V was changed to 0 mV. The increased time resolution of the current recording
mucosal bathing solution, and the transepithelial voltage (\(V_t\)) of the rabbit urinary bladder epithelium. Colistin was added to the mucosal bathing solution, and the transepithelial voltage (\(V_t\)) was held at −70 mV (serosal solution ground). At time 0, the \(V_t\) was changed to 0 mV. Note that there was a rapid increase in \(G_t\). The rate of conductance change was determined by fitting a linear equation to the initial linear portion of the conductance increase. In this example, the conductance increase was linear for ~4 s. To measure the increase in conductance with increased time resolution, we recorded the time course of the current recording demonstrates that there is a delay between the change in voltage to 0 mV and an increase in current.

To measure the increase in conductance with increased time resolution, we recorded the time course of the colistin-induced increase in transepithelial current at intervals of 5 ms. In brief, the protocol was to first measure the transepithelial current when \(V_t\) was changed from −70 mV to 0 mV in the absence of bath colistin. Next, the transepithelial current (in the presence of colistin) was recorded when \(V_t\) was changed from −70 mV to 0 mV. The difference between the colistin-induced current and control current is then the time-dependent increase in the colistin-induced current. Figure 1B is an example of the colistin-induced current. In this example, there is a delay of ~115 ms followed by an increase in current that is linear after ~800 ms. This delay in the increase in current after the voltage was changed from −70 mV to 0 mV suggests that there are a number of kinetic steps before colistin increases the current flow across the epithelium.

### Site of Action

The above data demonstrate that colistin increases \(G_t\) in a time- and voltage-dependent manner. The method of Yonath and Civan (19) was used to determine whether colistin was altering the conductance of the tight junctions or the cell membrane. If colistin is increasing the conductance of the tight junction, then \(G_t\) will increase in the absence of an increase in \(I_{sc}\). If colistin increases the cell membrane conductance, there will be a direct relationship between the increase in \(G_t\) and the increase in \(I_{sc}\). As shown in Fig. 2, the relationship between \(G_t\) and \(I_{sc}\) is linear, suggesting that colistin is increasing the apical membrane conductance. The inverse slope of a plot of \(G_t\) vs. \(I_{sc}\) is equal to the cell electromotive force \((E_c)\) and was −45.3 mV. Because the basolateral electromotive force \((EMF)\) is approximately −55 mV (9), the apical EMF is equal to 9.7 mV (cell interior ground). The ion selectivity of the colistin-induced conductance was determined from this apical membrane EMF, published intracellular ion activities (8), and the constant field equation. The chloride-to-potassium selectivity for the colistin-induced conductance is 1.26 ± 0.44 (n = 6). This suggests that colistin introduces a nonselective conductance at the apical of the urinary bladder epithelium.
Voltage Dependence

An interesting observation is that when the apical membrane potential is clamped such that the cell interior is made electrically positive, colistin does not cause an increase in the membrane potential. In contrast, when the apical membrane voltage is made cell interior negative, there is a large increase in the membrane conductance. The relationship between membrane voltage and induced conductance is shown in Fig. 3. The smooth curve through the data points is the best fit of Eq. 3 to the data

\[ \Delta G_{t}^{\text{Col}}(V) = \Delta G_{t}^{\text{Col}}(0) \exp^{(mV/RT)} \]  

where \( \Delta G_{t}^{\text{Col}}(V) \) is the rate of increase of the colistin conductance as a function of voltage; \( \Delta G_{t}^{\text{Col}}(0) \) is the rate of increase in conductance at zero voltage; \( V \) is voltage; \( R, T, \) and \( F \) have their usual meanings, and \( m \) is an empirical factor that determines the steepness of the rise of the exponential. The best-fit value for \( m \) at 100 \( \mu \)M colistin is 1.52 ± 0.33 (\( n = 4 \)). The value for \( m \) at 40 \( \mu \)M colistin (2.08 ± 0.2; \( n = 5 \)) is not significantly different from that at 100 \( \mu \)M colistin (\( P = 0.18 \)).

This voltage sensitivity for colistin is similar to that reported for other cationic proteins (15, 16).

Colistin Concentration and \( G_t \)

Most of the previous experiments were performed by using 40 \( \mu \)M colistin. We next determined the dependence of the rate of conductance change to the bath concentration of colistin. The experimental protocol was to voltage clamp the epithelium to \(-70 \) mV, add colistin to the mucosal solution, and, after a 5-min incubation, voltage clamp the epithelium to 0 mV. After the initial rate of increase of \( G_t \) was measured, the voltage was clamped back to \(-70 \) mV. When \( G_t \) had decreased to baseline, a higher concentration of colistin was added to the mucosal solution. After a 5-min incubation, the voltage was clamped to 0 mV, the rate of conductance increase was measured, the voltage was clamped to \(-70 \) mV, and \( G_t \) was allowed to decrease to baseline. This procedure was repeated until a complete dose-response curve was recorded. The concentration-conductance curve for colistin was sigmoidal (Fig. 4), suggesting that colistin may associate with multiple binding sites before it can alter the apical membrane conductance. The data were fit by a kinetic scheme for multiple, highly cooperative sites (Hill equation)

\[ \Delta G_{t}^{\text{Col}}(\text{Col}) = \frac{\Delta G_{t}^{\text{Col}}(\text{max})}{1 + \left( \frac{K_{\text{Col}}}{[\text{Col}]} \right)^N} \]  

where \( \Delta G_{t}^{\text{Col}}(\text{Col}) \) is the rate of conductance change at a given concentration of colistin, \( \Delta G_{t}^{\text{Col}}(\text{max}) \) is the maximum rate of colistin-induced conductance change, \( K_{\text{Col}} \) is an apparent dissociation constant, and \( N \) is the number of sites. The best-fit value for \( K_{\text{Col}} \) was 272 ± 48 \( \mu \)M (\( n = 6 \)), \( \Delta G_{t}^{\text{Col}}(\text{max}) \) was 275 ± 73 \( \mu \)S cm\(^{-2} \) s\(^{-1} \) (\( n = 6 \)), and \( N \) was 2.1 ± 0.24 (\( n = 6 \)).

Effect of Divalent Cations

In previous studies it was demonstrated that millimolar concentrations of Ca\(^{2+} \) or Mg\(^{2+} \) reduce the magnitude of conductance change caused by cationic proteins (16). This effect of divalent cations can occur by three different mechanisms: rapid and reversible block of the conductance after it is induced (conductive block), rate of reversal (divalent cations alter the time course of the reversal of the colistin-induced conductance), and competition for a colistin membrane binding site. We investigated each of these possibilities in turn.

Conductive block. To determine whether Ca\(^{2+} \) or Mg\(^{2+} \) are conductive blockers, we used the standard protocol (see Effect of Colistin on \( G_t \)). After colistin had increased the conductance by \( \sim 200 \) \( \mu \)S cm\(^{-2} \), it was removed from the mucosal bath, and...
is not significantly different from zero. This suggests that Ca\(^{2+}\) completely blocks the colistin-induced conductance in a reversible manner. The best-fit value for the Mg\(^{2+}\) \(K_i\) was 8.6 ± 1.4 mM \((n = 5)\), with Mg\(^{2+}\) blocking 72 ± 3% of the colistin-induced conductance. The fraction of colistin-induced conductance not blocked by Mg\(^{2+}\) \((27\%)\) is significantly different from zero. Thus Mg\(^{2+}\) cannot completely block the colistin-induced conductance. A possible explanation for this observation is that there are two populations of conductances, only one of which is blocked by Mg\(^{2+}\), whereas both are blocked by Ca\(^{2+}\).

**Rate of reversal.** The effect of divalent cations on the rate of reversal was studied by using the following protocol. After colistin had increased the transepithelial conductance by 200–300 μS/cm\(^2\), the mucosal solution was replaced with a colistin-free solution and the time course of the conductance decrease was followed. A kinetic model (originally developed for PMB) was used to describe the time course of colistin reversal \((2)\). In brief, when colistin is removed from the bath, a membrane-associated (nonactive) pool of colistin can either dissociate from the membrane and enter the bath, or it can form a membrane conductance \((G_{\text{Col-S}})\). This active membrane conductance can leave the membrane with a rate constant \(k_{\text{ao}}\) or enter a stable conductive state \((G_{\text{Col}})\) with a rate constant of \(k_{\text{as}}\). If the colistin-induced conductance is completely reversible, then \(k_{\text{as}}\) will be zero. Figure 6 shows that at a holding voltage of 0 mV and in the absence of mucosal Ca\(^{2+}\) or Mg\(^{2+}\), the colistin-induced conductance is only partially reversed. The best-fit value for \(k_{\text{ao}}\) is 0.015 ± 0.004 s\(^{-1}\), and that for \(k_{\text{as}}\) is 0.004 ± 0.001 s\(^{-1}\) \((n = 7)\). Figure 7 shows the effect of bath Ca\(^{2+}\) and Mg\(^{2+}\) on the rate constant for the active conductance leaving the membrane \((k_{\text{ao}})\) or entering the stable state \((k_{\text{as}})\). Of

![Graph](image_url)

**Fig. 5.** Effect of Ca\(^{2+}\) concentration on the colistin-induced conductance. After conductance was induced with colistin (using the standard protocol), the colistin was washed out of the lumen. Increments of Ca\(^{2+}\) were added to the luminal solution in a stepwise manner. The resulting decrease in \(G_{\text{Col}}\) by Ca\(^{2+}\) was then fit by the Michaelis-Menten equation. In this example, the best-fit \(K_i\) was 0.85 mM, and Ca\(^{2+}\) inhibited 86% of the colistin-induced conductance. \(G_{\text{Col}}\) is then shown in Fig. 5. The data were fit by the inhibition form of the Michaelis-Menten equation. The best-fit value for the Ca\(^{2+}\) \(K_i\) was 0.81 ± 0.12 mM \((n = 10)\), with Ca\(^{2+}\) blocking 85 ± 6% of the colistin-induced conductance. The fraction of the colistin-induced conductance that was not blocked by Ca\(^{2+}\) \((15\%)\) then either Ca\(^{2+}\) or Mg\(^{2+}\) was added to the mucosal bath from a concentrated stock solution. Addition of either Ca\(^{2+}\) or Mg\(^{2+}\) resulted in a rapid decrease in the colistin-induced conductance. The relationship between the magnitude of the conductance decrease and concentration of either Ca\(^{2+}\) or Mg\(^{2+}\) is shown in Fig. 5. The data were fit by the inhibition form of the Michaelis-Menten equation. The best-fit value for the Ca\(^{2+}\) \(K_i\) was 0.81 ± 0.12 mM \((n = 10)\), with Ca\(^{2+}\) blocking 85 ± 6% of the colistin-induced conductance. The fraction of the colistin-induced conductance that was not blocked by Ca\(^{2+}\) \((15\%)\)
interest is that both rate constants are insensitive to Ca\(^{2+}\) and Mg\(^{2+}\).

**Binding site.** The magnitude of the colistin-induced conductance saturates at high colistin concentrations (Fig. 4). This suggests that there is a membrane binding site for colistin. To determine the affinity of Ca\(^{2+}\) and Mg\(^{2+}\) for the binding site, the standard experimental protocol was employed, using a constant bath colistin concentration at different bath divalent cation concentrations. With the use of the values from Fig. 5, the rate of colistin-induced conductance increase as a function of bath divalent cation concentration was corrected for conductive block, and this corrected conductance is \( \Delta G_{\text{t,Col}}^\text{Col} (X) \). The corrected data were then fit by a kinetic model that describes the binding of colistin or a divalent cation to a common binding site. The equation that describes the effect of divalent cations on \( \Delta G_{\text{t,Col}}^\text{Col} (X) \) is

\[
\Delta G_{\text{t,Col}}^\text{Col} (X) = \frac{\Delta G_{\text{t,Col}}^\text{Col} (\text{max})}{K_X [X] + K_{\text{Col}} \frac{[\text{Col}]}{[\text{Col}]}}
\]

where \( K_X \) is the dissociation constant of a divalent cation from the binding site and \( K_{\text{Col}} \) is the dissociation constant of colistin from the binding site (272 \( \mu \)M). [Col] is the bath colistin concentration, [X] is the divalent cation concentration, and \( \Delta G_{\text{t,Col}}^\text{Col} (\text{max}) \) is the maximum rate of colistin-induced conductance change. Figure 8 shows the relationship of normalized \( \Delta G_{\text{t,Col}}^\text{Col} (X) \) (normalized to 200 \( \mu \)M colistin) as a function of the divalent cation concentration. Although both divalent cations seem to compete with colistin for a membrane-binding site, the affinity of this site for Ca\(^{2+}\) (\( K_X = 0.19 \pm 0.06 \) mM, \( n = 6 \)) is significantly higher than that for Mg\(^{2+}\) (\( K_X = 1.37 \pm 0.24 \) mM, \( n = 6 \)). The best-fit normalized \( \Delta G_{\text{t,Col}}^\text{Col} (\text{max}) \) for Ca\(^{2+}\) was \( 2.6 \pm 0.06 \) (\( n = 6 \)), and that for Mg\(^{2+}\) was \( 2.5 \pm 0.01 \) (\( n = 6 \)). These numbers are in reasonable agreement with the value of 2.4, calculated from Eq. 4 for the conditions of zero divalent cations, a colistin concentration of 200 \( \mu \)M, and a \( K_{\text{Col}} \) of 272 \( \mu \)M.

Fig. 7. A: effect of Ca\(^{2+}\) on the reversal of the colistin-induced conductance. The experimental protocol and data analysis were similar to those described in Fig. 6, except that the luminal bath contained known concentrations of Ca\(^{2+}\). B: same as in A, except that the luminal bath contained known concentrations of Mg\(^{2+}\). Of interest is that divalent cations did not alter either \( k_{ao} \) or \( k_{as} \).

**Fig. 8.** A: effect of luminal Ca\(^{2+}\) on \( \Delta G_{\text{t,Col}}^\text{Col} \), which has been corrected for conductive block. The standard experimental protocol was used, except that the initial rate of colistin-induced conductance was measured with known concentrations of luminal bathing solution Ca\(^{2+}\). The corrected data were fit to Eq. 5. The affinity of Ca\(^{2+}\) for the membrane binding site is \( 0.19 \pm 0.06 \) mM (\( n = 6 \)). B: effect of luminal Mg\(^{2+}\) on \( \Delta G_{\text{t,Col}}^\text{Col} \); the data have been corrected for conductive block. The standard protocol was used, except that the initial rate of conductance increase was measured in the presence of known concentrations of luminal bathing solution Mg\(^{2+}\). The corrected data were fit to Eq. 5. The colistin concentration was 200 \( \mu \)M. The affinity of Mg\(^{2+}\) for the binding site is \( 1.37 \pm 0.24 \) mM (\( n = 6 \)).
Reversal

We have demonstrated that removal of colistin from the mucosal bath at 0 mV resulted in a partial reversal of the colistin-induced conductance. In addition, Ca$^{2+}$ or Mg$^{2+}$ did not alter the time course of the conductance decrease or the fraction of the colistin-induced conductance that remained in the membrane (when $V_t$ was held at 0 mV, ~15%).

In the presence of bath colistin (but the absence of bath Ca$^{2+}$ and Mg$^{2+}$), the colistin-induced conductance can be reversed by changing $V_t$ from 0 mV to ~70 mV. The time course of the reversal was composed of a rapid decrease in $G_t$ (in less than 1 s), followed by a slow decrease to the pre-colistin levels (Fig. 9). The slow decrease was described by an exponential function with a time constant of $0.082 \pm 0.021$ s$^{-1}$ ($n = 6$). Thus, at ~70 mV and in the presence of bath colistin, the colistin-induced conductance is completely reversed. The ratio of the colistin-induced conductance immediately after $V_t$ was changed from ~70 mV to the colistin-induced conductance at 0 mV was $0.20 \pm 0.04$ ($n = 12$) in the presence of 200 $\mu$M colistin. Thus, immediately after $V_t$ changed from 0 mV to ~70 mV, there was an 80% decrease in the colistin-induced conductance. The ratio of colistin-induced conductance at ~70 mV and 0 mV ($G_{70}/G_0$) is dependent on the mucosal bath composition (Table 2). Table 2 also shows the ratio of the fast conductance change to slow conductance change ($G_{slow}/G_{slow}$). Thus, in the absence of bath colistin, $G_{70}/G_0$ is close to unity and $G_{fast}/G_{slow}$ is close to zero; i.e., in the absence of bath colistin, there is no fast component. In the presence of colistin and 2 mM Ca$^{2+}$ or 1.2 mM Mg$^{2+}$, the ratio is 0.58.

The effect of Ca$^{2+}$ or Mg$^{2+}$ concentration on the conductance ratio was determined by using the following protocol. First, with $V_t$ held at ~70 mV, either Ca$^{2+}$ or Mg$^{2+}$ was added to the mucosal bath, followed by 200 $\mu$M colistin. After a 5-min incubation, the voltage was changed to 0 mV, the conductance was allowed to increase by ~100 $\mu$S/cm$^2$, and the voltage was then changed to ~70 mV and $G_t$ recorded. Figure 10 shows the effect of Ca$^{2+}$ and Mg$^{2+}$ on $G_{fast}/G_{slow}$. Of interest is that Ca$^{2+}$ has a greater effect on the conductance ratio than Mg$^{2+}$.

Long-Term Exposure

Although the colistin-induced conductance can be completely reversed by changing the voltage from 0 mV to ~70 mV, this is only true for short exposure times. In this experiment the tissues were exposed to colistin in Ca$^{2+}$-, Mg$^{2+}$-, or Ca$^{2+}$- and Mg$^{2+}$-containing KCl Ringer and held at 0 mV for a specified period of time, and then colistin was removed from the bath. $V_t$ was changed to ~70 mV, and the new steady-state conductance was recorded. The longer the tissue was exposed to colistin at 0 mV, the greater was the new steady-state conductance (Fig. 11). Thus colistin has produced an irreversible change in the barrier function of the bladder epithelium. After 120 min of exposure, three of the four tissues did not reach a new steady-state conductance; i.e., $G_t$ continued to increase. The continued increase in conductance after the removal of colistin suggests that long-term exposure to colistin is toxic to the bladder epithelial cells.

DISCUSSION

The data presented in this paper strongly suggest that colistin increases the transepithelial conductance of the rabbit urinary bladder epithelium. The magnitude of the conductance increase was dependent on concentration, length of exposure, voltage, and divalent cation concentration. At low concentrations, for short exposure periods and at a $V_t$ of 0 mV, the removal of colistin from the bath resulted in an almost complete return of the conductance to control values. In contrast, increasing exposure time and colistin concentration resulted in an incomplete reversal of the induced conductance. Because the induced conductance is nonselective for small monovalent cations and anions, a possible mechanism for the irreversible nature of colistin is an increase in paracellular conductance caused by an influx of cations and anions producing cell swelling and cell lysis. The presence of Ca$^{2+}$ or Mg$^{2+}$ in the bath decreased the

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<th>[Colistin], $\mu$M</th>
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<td>3</td>
<td>*</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0</td>
<td>5.6±1.3</td>
<td>0.20±0.04</td>
<td>12</td>
<td>*</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>0</td>
<td>2.6±0.5</td>
<td>0.29±0.04</td>
<td>3</td>
<td>*</td>
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<tr>
<td>0</td>
<td>2</td>
<td>1.2</td>
<td>0.045±0.02</td>
<td>1.1±0.02</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>2</td>
<td>1.2</td>
<td>0.82±0.02</td>
<td>0.58±0.05</td>
<td>7</td>
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</tr>
</tbody>
</table>

For 0 $\mu$M colistin, the conductance was induced with 200 $\mu$M colistin (for calcium- and magnesium-free solutions) or 400 $\mu$M colistin (for calcium- and magnesium-containing solutions) at 0 mV. Colistin was then removed from the mucosal solution, and the voltage was changed to ~70 mV. The conductance ratio ($G_{70}/G_0$) was calculated by using the last measured conductance at 0 mV (minus the baseline conductance) and the first measured conductance at ~70 mV (minus the baseline conductance). These 2 values are measured less than 1 s apart. The ratio of the fast conductance change to slow conductance change ($G_{fast}/G_{slow}$) was calculated as ($G_0 - G_{70}$)/$G_{70}$. Values for ratios are means ± SE; n = no. of experiments. *Significantly different ($P < 0.05$) from 0 $\mu$M colistin (in the presence or absence of calcium and magnesium in the solution). †Significantly different ($P < 0.05$) from colistin-containing and calcium- and magnesium-free solutions.
increase might be due to the direct effect of colistin or might be a secondary effect, e.g., disruption of tight junctions. The initial rate of the conductance increase for either of the exponentials was similar to the rate of increase of the rapid linear increase. The delay suggests that the voltage-dependent formation of the conductance is a multiple step process with a series of nonconductive steps. The relationship between the initial rate of conductance increase and colistin concentration was sigmoidal, with a Hill coefficient of 2. This suggests that two molecules of colistin are required to induce a conductance.

After the holding voltage was changed from 0 mV to −70 mV, there was a rapid decrease in the conductance, followed by a slower decrease. The rapid decrease required the presence of colistin in the bath (Table 2). In the presence of colistin, increasing bath Ca2+ or Mg2+ reduced the magnitude of the rapid decrease of the conductance (see Fig. 10). Speculation on the mechanism of the voltage sensitivity described above is not warranted until a molecular model of how colistin forms a membrane conductance is developed.

Comparison of PMB and Colistin

Table 3 compares some of the properties of PMB (1, 2) and colistin (present study). Similarities between PMB and colistin include voltage sensitivity, anion/cation selectivity, conductive block by Ca2+ and Mg2+, affinity of Ca2+ and Mg2+ for the membrane binding site, Hill coefficient, and reversal of the induced conductance by voltage and washing. This finding suggests that the basic molecular mechanisms by which these two molecules induce a conductance are the same.

There are a number of differences between PMB and colistin. Some of the differences are minor, such as the dissociation constant of Mg2+ conductive block, the ability of Ca2+ to reverse the induced conductance at 0 mV, the rate of reversal of Mg2+ in the absence of Ca2+ or Mg2+, and the degree of magnitude of the colistin-induced conductance. This Ca2+ or Mg2+ effect had two sites of action: one was a reversible block of the colistin-induced conductance, and the second was competitive interaction at a membrane binding site.

Colistin was found to increase the apical membrane conductance only when the apical membrane voltage was cell interior negative. The time course of the increase (induced by 40 μM colistin) had a short delay followed by one of three different responses: an exponential, the sum of an exponential and a very slow linear increase, or a rapid linear increase. Ninety-one percent of the time courses contained an exponential. This finding suggests that, at steady state, there is an equal rate of conductance formation and dissociation. The very slow linear

Fig. 10. A: effect of Ca2+ on the ratio of the rapid decrease in colistin-induced conductance to the slow decrease (Gfast/Gslow) when the voltage was changed from 0 mV to −70 mV. The standard protocol was used, except that known concentrations of Ca2+ were in the luminal bathing solution. The luminal bathing solution colistin concentration was 200 μM. After Gt had increased, Vl was changed back to −70 mV and Gt was measured within 1 s. Note that increasing Ca2+ concentration decreased the ratio of the fast conductance change to the slow conductance change. B: effect of Mg2+ on the ratio of the colistin-induced conductance. The experimental protocol was similar to that in A, except that known concentrations of Mg2+ were in the luminal bathing solution. The effect of Mg2+ on the conductance ratio is less than the effect of Ca2+.

Fig. 11. Long-term effect of colistin on the Gt of the urothelium. The standard protocol was used, except that the luminal bathing solution was a Ca2+- and Mg2+-containing KCl Ringer and that 200 μM colistin was added to the luminal bathing solution. Vl was changed to 0 mV for 15, 30, 60, or 120 min and then changed back to −70 mV, and the Gt was allowed to reach a steady-state value. Note that the longer the tissue was held at 0 mV, the higher was the new steady-state conductance. In 4 instances (solid circles with arrows), the conductance initially decreased when Vl was changed to −70 mV but then started to increase in an irreversible manner.
Conductive block

<table>
<thead>
<tr>
<th>Conductive block</th>
<th>Polymyxin B</th>
<th>Colistin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td></td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>No effect</td>
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Voltage sensitivity

<table>
<thead>
<tr>
<th>Voltage sensitivity</th>
<th>Polymyxin B</th>
<th>Colistin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mV, Wash</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction remaining</td>
<td>0.19</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>k_on, s⁻¹</td>
<td>0.026 ± 0.002</td>
<td>0.015 ± 0.004</td>
<td>0.03</td>
</tr>
<tr>
<td>k_off, s⁻¹</td>
<td>0.006 ± 0.001</td>
<td>0.004 ± 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>~70 mV, No wash</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G~ G₀</td>
<td>0.35 ± 0.02</td>
<td>0.18 ± 0.12</td>
<td>0.012</td>
</tr>
<tr>
<td>k_on, s⁻¹</td>
<td>0.054 ± 0.029</td>
<td>0.08 ± 0.021</td>
<td>0.021</td>
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Divalent cation effect

<table>
<thead>
<tr>
<th>Divalent cation effect</th>
<th>Polymyxin B</th>
<th>Colistin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversal, 0 mV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Complete</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>No effect</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Polymyxins are a class of cationic peptide antibiotics with at least 11 members. The two best-studied polymyxins are PMB and colistin. Structurally, PMB and colistin are similar; they are decapetides that have five positively charged amino acids (diaminobutyric acid) and contain one of two fatty acid tails. The relative proportion of the fatty acid tails for PMB is 65% 6-methylheptanoic acid (PMB1) and 35% 6-methylheptanoic acid (PMB2) (6), and colistin is 70% 6-methylheptanoic acid (colistin A) and 30% 6-methylheptanoic acid (colistin B) (3). The only difference is that PMB contains a D-phenylalanine at position 6, whereas colistin contains a D-leucine. This difference results in PMB being more bactericidal than colistin at low concentrations, whereas at high concentrations they have equivalent bactericidal activity (5, 10).

Results from the present study suggest that the increased activity of PMB at low concentrations is due to a higher binding affinity of PMB compared with colistin. Viljanen and colleagues (18) compared the binding of the peptide portion of PMB and colistin to bacterial membranes and found that PMB had a higher binding affinity. Synthesis of PMB nonapeptide (PMBN, a PMB lacking the fatty acid tail and terminal amino acid) in which D-phenylalanine was replaced with L-phenylalanine increased the dissociation constant (decreased the binding affinity) to bacterial lipopolysaccharide (by a factor of 3) (14). These authors concluded that the configuration of the phenylalanine is important for membrane association. Recent studies in which PMBN was used have led to the proposal that, in addition to the electrostatic binding activity of PMBN, there is an important interaction of the hydrophobic amino acids (D-phenylalanine-leucine) of the PMBN with the hydrophobic lipid environment of the bacterial membrane (13). Structural studies and molecular modeling (11) of PMB interaction with lipid A suggest that there is a deep penetration of D-phenylalanine-leucine into the hydrophobic core of lipid A, resulting in an increased hydrophobic interaction. Similar studies for colistin suggested that there is less penetration of leucine-leucine side chain into the lipid A hydrophobic core, resulting in reduced hydrophobic interactions (11). The affinity of polymyxin to a cell membrane is due to a hydrophobic interaction of the tail with the lipid membrane, an electrostatic interaction of the five positive charges with the negatively charged lipid membrane, and, last, an interaction of the hydrophobic portion of the heptapeptide ring with the lipid membrane. The last of these three interactions produces a higher binding affinity for PMB compared with colistin.

Although PMB has a higher binding affinity than colistin, the maximal rate of conductance change is less. In the most simple model, the maximal rate of conductance change is equal to the product of the number of conductive units formed per unit of time (this is equal to rate of formation of conductive units minus rate of loss), the conductance of a single unit, and the open probability of a single unit. The data in this study do not allow one to differentiate among these possibilities.

In summary, colistin and PMB share many features in common, including voltage sensitivity, a membrane binding site for which they compete with divalent cations, and divalent cation block. They differ in their dissociation constant (PMB has a lower value than colistin) and the maximum rate at which they induce a membrane conductance change (at saturating

Table 3. Comparison of polymyxin B and colistin

<table>
<thead>
<tr>
<th>Time course</th>
<th>Polymyxin B</th>
<th>Colistin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage sensitivity</td>
<td>Linear</td>
<td>Exponential</td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>1.24 ± 0.16</td>
<td>1.52 ± 0.33</td>
<td>NS</td>
</tr>
<tr>
<td>Selectivity</td>
<td>PMB/Col</td>
<td>1.7 ± 0.4</td>
<td>1.26 ± 0.44</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Kₘ, µM</td>
<td>66</td>
<td>272 ± 48</td>
</tr>
<tr>
<td>Conductivity</td>
<td>N</td>
<td>1.7</td>
<td>2.1 ± 0.24</td>
</tr>
<tr>
<td>Conductivity</td>
<td>ΔGᵢ(max), µS cm⁻² s⁻¹</td>
<td>156</td>
<td>275 ± 73</td>
</tr>
</tbody>
</table>
concentrations, colistin induces a greater rate of membrane conductance change. These differences are due to a single amino acid. The lower dissociation constant for PMB compared with colistin can be explained by a stronger hydrophobic interaction between the heptapeptide ring and the lipid environment of the membrane. The greater rate of conductance change can be due to differences in the rate of formation of the conductance and to the size or open probability of an individual conductive unit. The lower dissociation constant for PMB is in agreement with the observation that PMB is more bactericidal at low concentrations than colistin, but at high concentrations they are similar.

GRANTS
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-51382.

REFERENCES