Regulation of Tight Junction Permeability by Calcium Mediators and Cell Cytoskeleton in Rabbit Tracheal Epithelium

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Received October 7, 1992; accepted December 21, 1992

The present study investigates the mechanisms controlling tight junction permeability of the tracheal epithelium, with an emphasis on the regulatory role of intra- and extracellular calcium as well as the cell cytoskeleton. The tracheas were isolated from rabbits and their junctional permeability barrier was investigated *in vitro* by means of transepithelial electrical resistance measurements and flux measurements of the radiolabeled paracellular tracer, ^{14}C-mannitol. The effects of intra- and extracellular calcium were studied using the calcium ionophore A 23187 and EGTA, and that of the cytoskeleton was investigated using cytochalasin B. Intracellular calcium of the tracheal epithelium was monitored microfluorometrically using the specific calcium indicator, Fura-2 AM (acetoxymethyl ester). The results indicate that the tight junction permeability of the trachea was significantly increased upon treatment with all three of the test compounds, as evidenced by a substantial decrease in transepithelial electrical resistance and an increase in transepithelial flux of ^{14}C-mannitol. The effects of EGTA and cytochalasin B on the tight junction permeability are fully reversible upon removal of the compounds from the bathing media. On the other hand, tissues treated with the calcium ionophore demonstrate a partial or no recovery in membrane permeability, depending on the intracellular calcium levels. Moderate and transient increases in intracellular calcium caused a partial reversibility of the membrane resistance, while high and sustained intracellular calcium levels induce a complete irreversibility of the membrane resistance. These results suggest that high extracellular calcium levels and low intracellular calcium levels are required for the normal maintenance of the junctional permeability in the tracheal epithelium. Studies using cytochalasin B indicate that there is also a close relationship between the tight junctions and the organization of actin microfilaments. Alterations of these structures as well as cellular calcium levels can result in a substantial change in transepithelial permeability. Therefore compounds that affect tight junction permeability may exert their action through the calcium and cytoskeleton mechanisms.

KEY WORDS: tracheal epithelium; paracellular; tight junction permeability; calcium; cytoskeleton.

INTRODUCTION

The use of the lung as a potential site for the systemic delivery of drug substances has gained considerable attention in recent years because of its favorable properties for drug absorption, which include a large absorptive surface area, an extensive microcirculation, and a rapid distribution of the therapeutic agents in the airspaces following drug administration, i.e., inhalation therapy. Drugs that undergo first-pass metabolism or extensive GI proteolytic degradation, e.g., peptides and proteins, have been shown to demonstrate an improved drug bioavailability when administered through the respiratory route (1–3). Like most other epithelia, the epithelial lining of the lung provides significant resistance to drug absorption, although previous studies have indicated that the permeability barrier of the lung is relatively low, i.e., the relative permeabilities of the lung epithelia as compared to other epithelia are intestinal $\geq$ nasal $\approx$ tracheal $\approx$ bronchial $>$ vaginal $>$ rectal $>$ corneal $>$ buccal $>$ skin (4,5). In the lung, it is generally agreed that drug absorption occurs most extensively in the pulmonary region, although recent studies have suggested that significant drug absorption can also occur in the airway region, depending on the site of drug deposition and the accessibility of the drug in the deeper region following administration (6,7). In their recent studies, Vidgren *et al.* (6) demonstrated that more than 80% of the drug entering the lung following normal inhalation administration via a metered dose inhaler were deposited in the airway and only less than 10% actually entered the pulmonary region. Labedzki *et al.* (7) also showed that local administration of the anesthetic lidocaine to the airway epithelium resulted in a significant systemic drug absorption. Because drug absorption in the airway can have a significant influence on the overall transport of drugs in the lung and because the transport properties of the airway epithelium have not been well studied, the present study investigates its transport properties and its control mechanisms of tight junction permeability, using isolated tracheal epithelium as a model system.

Drug absorption across epithelial membranes can occur via two potential routes, the transcellular and paracellular routes. The transcellular route is important for absorption of lipophilic molecules or molecules that possess specific recognition of the membrane sites which allow transport by carrier- or receptor-mediated processes to occur. On the other hand, the paracellular pathway relies on a passive process, by which molecules have to diffuse across the intercellular junctional complexes of the membrane. The junctional complex consists of the tight junctions (zonulae occludens), intermediate junctions (belt desmosome or zonulae adherens), and spot desmosomes (maculae adherentes). These junctional structures present a major transport barrier for most hydrophilic drugs. The barrier properties of the junctional pathway in various epithelia have been shown to depend primarily on the degree of tightness of the tight junctions and can be quantitatively assessed by measurements of transepithelial electrical resistance (5). In the airway epithelium, an increase in tight junction permeability has been shown to promote paracellular penetration of hydrophilic macromolecules such as horseradish peroxidase (8). The tight junctions are dynamic structures which undergo constant assembly and disassembly in response to various physiological stimuli (9,10). The dynamic nature of these structures also suggests that the permeability barrier of the epithelia can be regulated by exogenous substances. Indeed,

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0724-8741/93/0700-0991$07.00/0

several externally applied agents such as anesthetic ethers, oxidants, calcium chelators, cytochalasins, and phorbol esters have been shown to increase the paracellular permeability of the respiratory epithelia (8,11,12). While these studies have demonstrated the ability of certain compounds to regulate the tight junction complex and thus the permeability barrier to flux of solutes, the underlying mechanisms as well as the reversibility of the effects of these agents remain unclear. The mechanism governing membrane reversibility is of particular interest for pharmaceutical scientists due to the potential usage in drug delivery, i.e., the membranes may be transiently modified to allow enhanced drug absorption without long-term undesirable side effects. Understanding of the underlying mechanisms would also allow development of more specific and perhaps more effective methods to promote drug absorption, in addition to gaining a better insight into the pulmonary functions.

In the present study, the potentially reversible effects of two key cellular regulators, calcium and the cytoskeleton, on the tight junction permeability of isolated rabbit tracheal epithelium are investigated. The effects of calcium, which may be mediated by both *intra-* and *extra*cellular calcium levels, are studied with the aid of the specific calcium regulators, A23187 and EGTA, respectively. The effect of cell cytoskeleton is studied using the cytoskeleton-active agent cytochalasin B. This compound is used because it acts specifically on the actin microfilaments, the cytoskeletal component that has been shown to affect tight junction permeability (13,14). Changes in tracheal permeability are monitored using a combined method based on transepithelial electrical resistance measurements and flux measurements of the radioactive paracellular tracer ^{14}C-mannitol. Intracellular calcium is measured by the dual-wavelength fluorescence microscopy with the aid of the specific calcium indicator, Fura 2-AM.

EXPERIMENTAL

Materials and Methods

Animals

Male albino New Zealand rabbits (Greenmeadows Rabbitry, PA), weighing 2.5–3.0 kg, were used throughout the studies. Lighting was maintained on a 12-hr on/off basis in the caging facility, and the animals were fed a regular diet with no restriction on the amount of food or water consumed.

In Vitro Perfusion Studies

Rabbits were sacrificed with an intravenous injection of an overdose of sodium pentobarbital given via the marginal ear vein. The tracheas were removed (≈2-cm^2 size) with a pair of fine scissors and placed in ice-cold HEPES-buffered medium, pH 7.4, containing 136 m*M* NaCl, 2.2 m*M* Na_2HPO_4, 5.3 m*M* KCl, 10 m*M* HEPES, 1.0 m*M* $CaCl_2$, and 5.6 m*M* glucose. Great care was taken to avoid any direct trauma and damage to the tissues. The underlying connective tissue was removed and these tissues were then divided into two pieces, for control and test treatments. Tissues were removed from the ice bath and immediately mounted on supporting rings with the epithelial side facing the donor half-cell and then clamped in Ussing chambers as described previously (15). Silicone grease was applied to the contact surfaces to prevent possible leakage of bathing fluid. Tissue preparation and the mounting process was normally completed within 10 min after removal. After the tissues were mounted, the chambers were filled with a bathing solution which was comprised of HEPES buffer or of HEPES buffer without calcium ions plus 1.0 m*M* EGTA. In studies designed to investigate the effect of intracellular calcium and cytoskeletal alteration, HEPES solutions containing either calcium ionophore A23187 (Molecular Probes Inc.) or cytochalasin B (Aldrich Co.) were added. All test agents were applied only on the donor side. For flux studies, the donor compartments were spiked with 10 μCi of ^{14}C-mannitol (New England Nuclear). A 100-μL aliquot of the donor compartment solution was obtained in order to measure the initial dose of radioactivity present. Aeration and circulation of the tissue bath were performed by means of bubbling with a mixture of 95% O_2 and 5% CO_2. A 30-min equilibration period was allowed prior to each experiment in order to establish a steady-state baseline condition, which was indicated by a stable electrical resistance value (typically in the range of 260–320 Ω · cm^2). All experiments were conducted at 37°C using a constant-temperature bath connected through a water jacket to the Ussing chambers. A 0.67-cm^2 area of the tissue was exposed to the donor and receptor compartments, each having a volume of 7 mL.

Electrical and Flux Measurements

Membrane electrical resistance was measured using a four-electrode Ag/AgCl system according to the method described previously (15). Two electrodes (located 1 cm from each side of the membrane) were connected to a high-input impedance microvoltmeter (Keithley 197) and used as potential determining electrodes. The other two electrodes (positioned 2 cm from each side) were used to inject current pulses. To avoid problems associated with membrane polarization, alternating sinusoidal currents (current density, 1–10 μA cm^2; frequency, 1 Hz), generated from a function generator (Simpson 422), were used. In all experiments, the anode was placed on the donor side and the cathode on the receptor side.

The effects of extracellular calcium depletion, intracellular calcium elevation, and cytochalasin B on the transepithelial electrical resistance were tested over a period of 90 min, with the resistance readings taken every 10–15 min. Their effects on membrane reversibility were tested by replacing the test solutions after a 30-min incubation with HEPES-buffered medium. To correct for the potential drop due to the solution resistance between the sensing electrodes and the membrane, measurements were carried out before each membrane resistance determination using the same bathing solution but without the membrane in the perfusion chamber. The actual membrane resistance was then obtained by subtracting the resistance obtained without the membrane from that determined with the membrane. In studies involving flux measurements, the amount of radiolabeled mannitol transported across the control and treated tissues was calculated. Aliquots of 1 mL were withdrawn from the

receptor compartment every 30 min and replaced with an equal volume of fresh medium. The amount of radioactivity in the aliquots was determined, after an addition of a scintillation cocktail (10 mL), in a liquid scintillation counter.

Dual-Wavelength Fluorescence Determination of Intracellular Calcium

Intracellular levels of calcium following ionophore treatment of the tracheal epithelium were determined by recording the change in Fura-2 fluorescence. Fura-2 belongs to a class of fluorescent calcium indicators whose excitation wavelength changes upon binding to calcium, resulting in a greater fluorescence (16). This allows for an excitation ratioing technique which eliminates variations in fluorescence intensity due to illumination intensity, emission collection efficiency, dye concentration, and cell thickness in the optical beam (16). In the present study, the membrane permeant acetoxymethyl ester derivative of Fura-2 (Molecular Probes Inc.) was used. The tissues were incubated with the dye at a 1 μM concentration in HEPES-buffered medium for 30 min at room temperature to allow ester hydrolysis to take place within the cells. The resulting hydrolytic product, Fura-2, is membrane impermeable and retained in the cells, thus allowing intracellular calcium determination. Changes in Fura-2 fluorescence upon binding with calcium were recorded under the Nikon Diaphot microscope. Excitation light was provided by two monochromators (Spex Industries) preset at 340 and 380 nm and selected in rapid alternation (100 Hz) by a rotating chopper mirror. Emitted light was collected through a 510-nm interference filter and photon counted in synchrony with the chopper. The ratio of fluorescence excited at 340 and 380 nm was determined and used to calculate calcium concentrations, according to the equation (16): $[Ca^{2+}]_i = K_d F_0 (R - R_0)/F_s (R_s - R)$, where R is the fluorescence ratio, F is the fluorescence measured at an excitation of 380 nm, subscripts 0 and s denote zero and saturated calcium conditions, and K_d is the effective dissociation constant for Fura-2, 224 nM, as determined previously (16). R_0 and R_s were determined with the aid of the membrane lysing agent, digitonin (10 μM), and the calcium chelator, EGTA (10 mM), respectively.

RESULTS AND DISCUSSION

Correlation Between Transepithelial Electrical Resistance and Solute Flux

Figure 1 shows that the transepithelial resistance of the tracheas is significantly decreased over time in the presence of EGTA, cytochalasin B, or calcium ionophore A 23187 at concentrations effectively affecting the tight junctions of the epithelium. No significant difference, however, was observed between the values of the treated samples ($P < 0.05$). In these studies, the average initial baseline value was found to be 289 ± 34 $\Omega \cdot cm^2$ ($n = 16$). This value is consistent with those previously reported (4,5). Concurrent measurements of flux of ^{14}C-mannitol also indicate that these calcium and cytoskeleton regulators caused an increase in epithelial permeability which is positively correlated with the reduction in the transepithelial resistance (Fig. 2). These results suggest that drug permeation across the tracheal epithelium

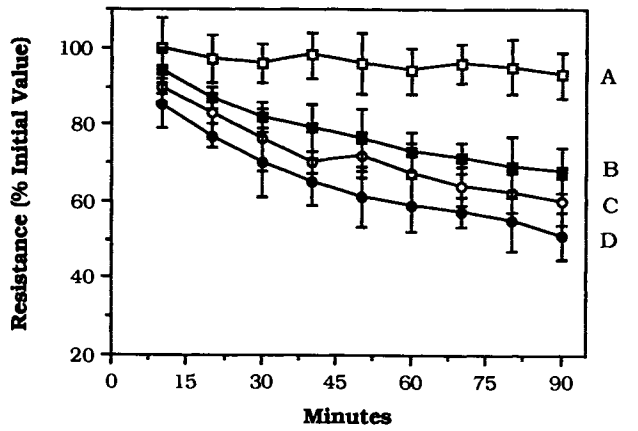

Fig. 1. Effect of calcium and cytoskeleton on transepithelial electrical resistance. The experiments were conducted *in vitro* using isolated rabbit tracheal epithelium in Ussing chambers at 37°C. The tracheas were incubated with HEPES-buffered media, pH 7.4, in the absence (A) or presence of 10 μM ionophore A 23187 (B), 0.1 mM cytochalasin B (C), or 1 mM EGTA (D). The electrical resistance was measured by applying a sinusoidal current (current density, 1–10 $\mu A \cdot cm^{-2}$; frequency, 1 Hz) across the tissue, and the potential drop and current passing through it were recorded through a four-electrode Ag/AgCl system, as described under Experimental. Bars indicate 1 SE; $n = 4$.

may be enhanced by mediators that affect the paracellular pathway. Although an increase in the transcellular transport by these mediators may not be disregarded, the permeability of this pathway to polar solutes such as mannitol and ionic species (as being measured during the electrical resistance determination) should be minimal.

Effect of Extracellular Calcium Deletion

The depletion of extracellular calcium by EGTA has been shown to increase tight junction permeability in a number of epithelia (17,18). The mechanisms by which the extracellular calcium affects the tight junctions are not yet clear. The effect is thought to be indirect since the isolated

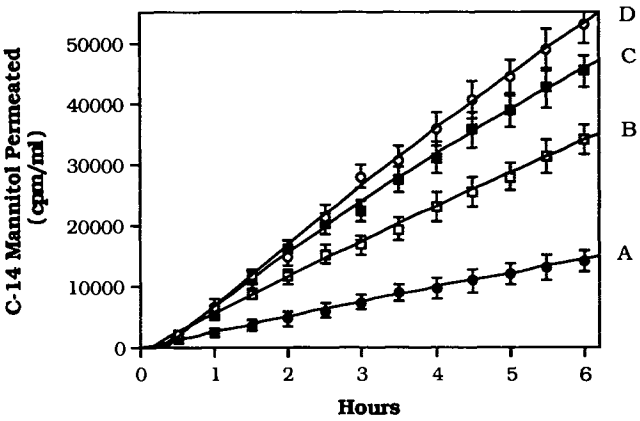

Fig. 2. Effect of calcium and cytoskeleton on transepithelial flux of C-14 mannitol. The tracheas were incubated with drug-free HEPES-buffered medium (A), or HEPES buffer containing 10 μM ionophore A 23187 (B), 0.1 mM cytochalasin B (C), or 1 mM EGTA (D). The figures indicate cumulative radioactivity appeared in the receptor chambers. Bars represent 1 SE; $n = 4$.

tight junction itself is not affected by a lack of calcium in the medium (19). In MDCK cell cultures and small intestinal epithelium, cell adhesion molecules such as uvomorulin have been noted to depend on calcium for their action (18). Uvomorulin is involved in the formation of the zonulae adherens, a region of cell–cell attachment below the tight junction. Thus, the zonulae adherens may provide the adhesion strength or localization necessary for the assembly of an organized tight junction. In addition, the effect of extracellular calcium may be mediated through the cytoskeletal structures. This is demonstrated by the observations that detachment of actin microfilaments from the junctional complex occurs at low extracellular calcium levels (18).

Effect of Cytochalasin B

Treatment of the tracheal epithelium with 0.1 m*M* cytochalasin B results in a decrease in transepithelial resistance and an increase in flux of mannitol over time (Figs. 1 and 2). In earlier studies using dual-flux techniques with ^{14}C-mannitol and $^{23}Na^{+}$, it was established that the increase in solute flux was solely due to changes in the paracellular permeability (20). This is probably due to the specific action of cytochalasin B on actin microfilaments (21). A similar response was seen in MDCK monolayers (17), guinea pig ileum (20), and T84 monolayers (22). The selectivity of cytochalasin B on the paracellular permeability was also confirmed electrophysiologically using a microelectrode technique, whereby the agent was found to interfere with the resealing of tight junctions (13). This increase in permeability was selective enough to discriminate between flux of differently sized solutes in T84 monolayers (22).

The relationship between actin microfilaments and junctional components is further supported by ultrastructural evidence which indicates a close association of microfilaments and microtubules with cellular junctions (13). However, earlier studies show that microtubules are not essential for junctional integrity (13). In the intestinal epithelium, actin microfilaments were found to associate with tight junctions through an electron-dense plaque (14). Cytochalasin B altered not only the pattern of microfilaments but also the freeze-fracture pattern of the occluding junctions. The same effect was seen in *Necturus* gallbladder epithelium (23).

The cytoskeleton may also influence tight junction structures by actin–myosin interactions (14). In cultured monolayers of mammary epithelia, application of mechanical tension results in altered tight junction structures (24). Other physiological factors or cellular events may lead to modification of tight junction properties via alterations in the cytoskeletal network, although this has not been definitely proven.

Effect of Calcium Ionophore A 23187

Figures 3 and 4 show that the exposure of the tissue to calcium ionophore A 23187 results in a dose-dependent increase in intracellular calcium of the epithelial cells and a corresponding decrease in the electrical resistance. The calcium levels increased from the resting 104 ± 21 n*M* to 213 ± 27 and 458 ± 41 n*M* (n = 4) following 5 and 10 μ*M* ionophore treatment, respectively. Ionophore at 1 μ*M* failed to induce

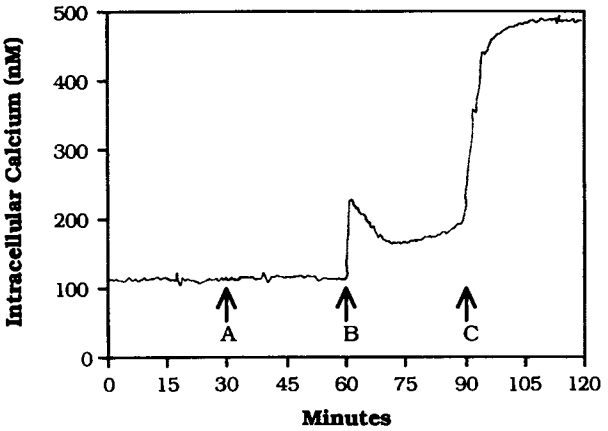

Fig. 3. Effect of calcium ionophore A23187 on intracellular calcium levels. The tracheal epithelial cells were loaded with Fura-2 AM (1 μ*M*) and then incubated in HEPES-buffered medium containing 1 μ*M* (A), 5 μ*M* (B), and 10 μ*M* (C) calcium ionophore A 23187. The arrows indicate the time of addition of ionophore. Intracellular calcium was calculated from the Fura-2 fluorescence ratio excited at 340 and 380 nm as described under Experimental. The trace is representative of four measurements obtained from different tissue preparations.

an elevated level of intracellular calcium or a change in membrane electrical resistance. The lack of cellular responses on both the calcium levels and the electrical resistance at a low dose (1 μ*M*) coupled with the corresponding changes observed at the two higher doses (5 and 10 μ*M*) suggests a close relationship between the two membrane parameters. A decrease in membrane electrical resistance following ionophore treatment was similarly reported in cultured MDCK epithelium (25), although the intracellular calcium was not determined in this study. In other studies using *Necturus* gallbladder epithelium, calcium ionophore was shown to cause a transient decrease and then an increase in membrane resistance (23). The decrease in the resistance in this study was attributed to the increase in the ionic conductance and

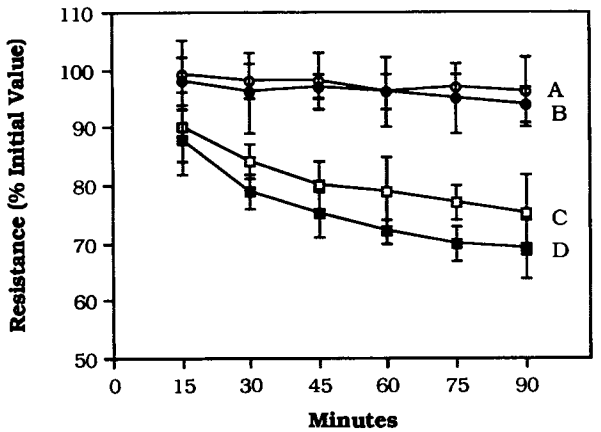

Fig. 4. Effect of intracellular calcium elevation on transepithelial electrical resistance. Isolated tracheal tissue were incubated with HEPES-buffered medium in the absence (A) or presence of 1 μ*M* (B), 5 μ*M* (C), or 10 μ*M* (D) calcium ionophore A 23187. Bars indicate 1 SE; n = 4.

hyperpolarization of this tissue. The subsequent increase in the resistance was attributed to the morphological disorganization of the tight junctions, which does not follow a dose–response pattern (23). The corresponding decrease in electrical resistance and increase in mannitol flux were also reported in Caco-2 monolayers following ionophore treatment (26). From these studies it appears, then, that intracellular calcium, like extracellular calcium, plays an important role in the regulation of tight junction permeability, although their effects are surprisingly opposite. Nonetheless, linkage between the two has been suggested, i.e., a decrease in extracellular calcium may induce intracellular calcium release from internal stores (e.g., mitochondria and endoplasmic recticulum) which is part of the normal calcium homeostatic mechanisms of the cells. In addition, the calcium effects may be operative through the cytoskeleton mechanism since the assembly and disassembly of the microfilaments are known to be regulated by calcium (21). Finally, the high-dose effect may also be associated with the potential cytotyoxic effect of the compound since a prolonged and sustained elevation of intracellular calcium following ionophore treatment has been shown to induce membrane damage in respiratory epithelia (29).

Reversibility Studies

Tight junctions are dynamic structures capable of rapid assembly and disassembly, either partially or completely, during many developmental and physiological processes. Electrophysiological experiments on *Necturus* gallbladder epithelium have shown that functional tight junctions can form very rapidly (18) and probably involve reassembly of junctional components since the process was not blocked by addition of inhibitors of protein synthesis. Our results show that the tight junction permeability of the tracheal epithelium is reversible upon removal and addition of extracellular calcium (Fig. 5A). These results are consistent with earlier work performed in cultured MDCK and Caco-2 epithelia (25,27). Martinez-Paloma *et al.* reported a simplification of the pattern of the tight junction strands upon removal of extracellular calcium (25). This effect was found to be reversible with the addition of calcium ions in the incubating medium (25).

Similar to EGTA, the effect of cytochalasin B (0.1 m*M*) on the tracheal permeability was found to be fully reversible, as evidenced by the recovery of the electrical resistance of the tissue (Fig. 5B). In the case of the ionophore, however, only a partial recovery was found (at a 5 μ*M* ionophore dose; see Fig. 5C). In all three cases, the effects of the test compounds on the transepithelial electrical resistance were significantly lower than the controls but not between the treated samples ($P < 0.05$). Higher doses of the ionophore (≥10 μ*M*) caused an irreversible decrease in membrane electrical resistance (data not shown). A partially irreversible increase in the electrical resistance was also shown in *Necturus* gallbladder epithelium (23). In Caco-2 monolayers, ionophore at doses >2.5 μ*M* was reported to cause an irreversible change in membrane permeability (26). The results of the present study further indicate the importance of intracellular calcium in its maintenance of normal cell function and membrane resistance. As can be seen in Figs. 3 and 5C, the

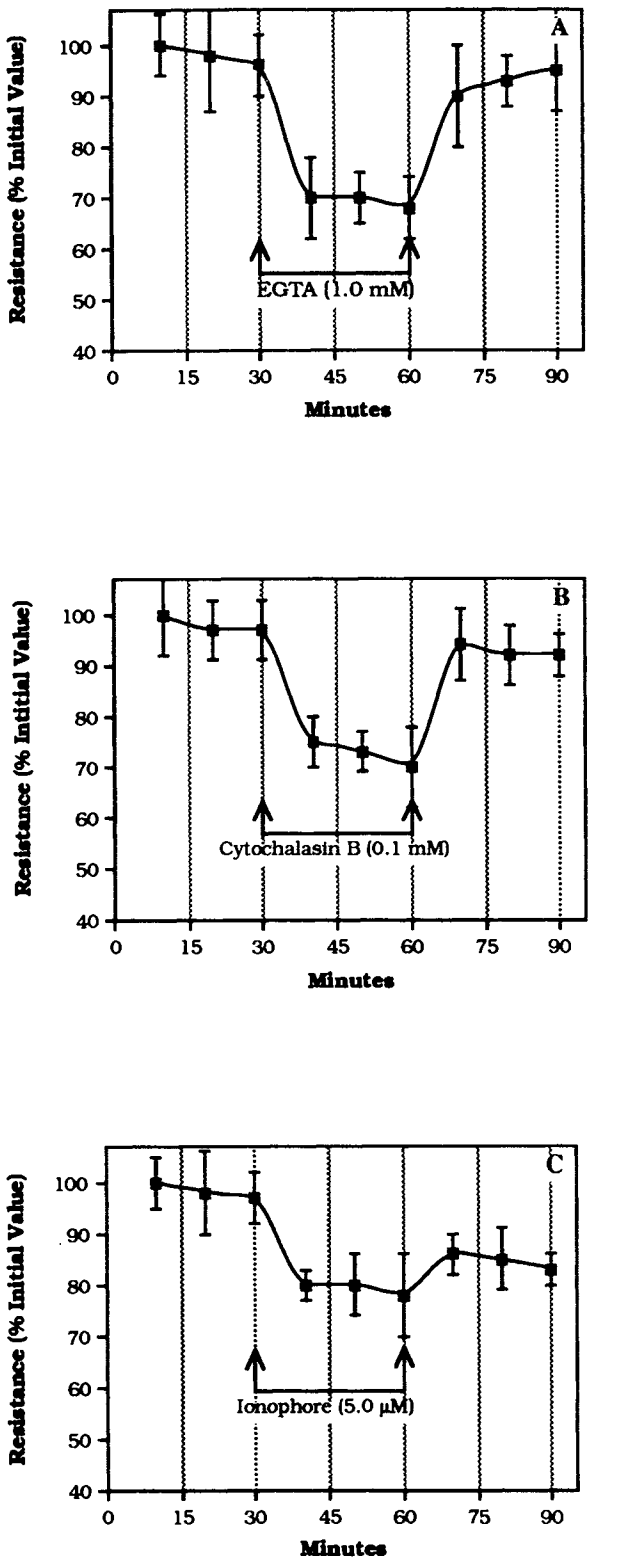

Fig. 5. Reversibility effects of calcium mediators and cytoskeleton on transepithelial electrical resistance. (A) The effect extracellular calcium depletion by calcium chelator EGTA (1 m*M*). (B, C) The effects of cytoskeleton and intracellular calcium alteration as induced by cytochalasin B (0.1 m*M*) and calcium ionophore A 23187 (5 μ*M*), respectively. The arrows indicate the time of addition and removal of the test agents. Bars represent 1 SE; $n = 4$.

reversible effect of calcium ionophore is associated with only a transient elevation of intracellular calcium. In conditions where sustained elevation of calcium occurs, the change in membrane resistance is irreversible. The mechanism through which elevated intracellular calcium exerts its effect on the junction permeability is incompletely understood but probably involves Ca^{2+}-dependent regulation of cytoskeleton, phospholipases, and proteases (28–30). The first can be demonstrated by the observations that elevated calcium levels cause a shortening of the actin filaments, the effect that was found to be operative through the Ca^{2+}-dependent severing action of the actin binding proteins (28). In this case, the irreversible decrease in membrane resistance associated with sustained elevation of intracellular calcium may be attributed to the impairment of the actin filaments and, thus, the dynamic nature of the tight junctions. Phospholipase activation with subsequent disruption of membrane integrity was also reported in respiratory epithelia following treatment with calcium ionophore (29). Although phospholipases can potentially affect membrane permeability, their effect is believed to be indirect, i.e., by general disruption of membrane structures rather than specific action on the junctional structures. The role of proteases on junctional permeability is relatively unknown, although several proteolytic enzymes have been shown to increase reversibly transepithelial transport of solutes such as sucrose and albumin in the tracheal epithelium (30).

CONCLUSIONS

The permeability barrier of the lung epithelium represents one of the major problems limiting the use of this tissue as the delivery site for the systemic application of therapeutic substances. The present study investigates the mechanisms controlling this permeability barrier in rabbit tracheal epithelium. Emphasis is given to the regulation of paracellular permeability due to its importance in absorption of hydrophilic drugs and presumably most peptides and proteins. Enhanced absorption of these compounds through the paracellular route may also provide additional advantage over transcellular absorption since proteolytic degradation, which would occur more extensively inside the cells, can be minimized. Actin microfilaments and calcium are key modulators of junction permeability in response to various physiological stimuli and exogenous substances. Permeability regulation through the mechanisms of extracellular calcium depletion, actin depolymerization, and transient elevation of intracellular calcium is shown to be reversible. High and sustained elevation of intracellular calcium as induced by the calcium ionophore at ≥ 10 μM causes irreversible change in membrane permeability, which may be a consequence of cell injury and cytoskeletal disruption due to altered calcium homeostasis. An understanding of the regulatory role and mechanisms of tight junction permeability and cell injury may provide useful strategies for the development of safe and effective systems for respiratory drug delivery.

ACKNOWLEDGMENT

This work was supported by a research grant from Rhone Poulenc Rorer and from the American Association of College of Pharmacy.

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