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Regulation of Tight Junction Resistance in T_{84} Monolayers by Elevation in Intracellular Ca^{2+}: A Protein Kinase C Effect

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Abstract. Elevation in intracellular Ca^{2+} acting via protein kinase C (PKC) is shown to regulate tight junction resistance in T_{84} cells, a human colon cancer line and a model Cl^- secretory epithelial cell. The Ca^{2+} ionophore A23187, which was used to increase the intracellular Ca^{2+} concentration, caused a decrease in tight junction resistance in a concentration- and time-dependent manner. Dual Na^+/mannitol serosal-to-mucosal flux analysis performed across the T_{84} monolayers treated with 2 μM A23187 revealed that A23187 increased both fluxes and that in the presence of ionophore there was a linear relationship between the Na^+ and mannitol fluxes with a slope of 56.4, indicating that the decrease in transepithelial resistance was due to a decrease in tight junction resistance. Whereas there was no effect of 0.1 μM A23187, 1 or 2 μM produced a 55% decrease in baseline resistance in 1 hr and 10 μM decreased resistance more than 80%. The A23187-induced decrease in tight junction resistance was partially reversible by washing 3 times with a Ringer's-HCO_3 solution containing 1% BSA. The A23187 effect on resistance was dependent on intracellular Ca^{2+}; loading the T_{84} cells with the intracellular Ca^{2+} chelator BAPTA significantly reduced the decrease in tight junction resistance caused by A23187. This intracellular Ca^{2+} effect was mediated by protein kinase C and not calmodulin. While the protein kinase C antagonist H-7 totally prevented the action of A23187 on tight junction resistance, the Ca^{2+}/calmodulin inhibitor W13 did not have any effect. Sphingosine, another inhibitor of PKC, partially reduced the A23187-induced decline in tight junction resistance. The PKC agonist PMA mimicked the A23187 effect on resistance, although the effect was delayed up to 1 hr after exposure. In addition, however, PMA also caused an earlier increase in resistance, indicating it had an additional effect in addition to mimicking the effect of elevating Ca^{2+}. The effects of a phospholipase inhibitor (mepacrine) and of inhibitors of arachidonic acid metabolism (indomethacin for the cyclooxygenase pathway, NDGA for the lipoxygenase pathway, and SKF 525A for the epoxygenase pathway) on the A23187 action were also examined. None of these agents altered the A23187-induced decrease in resistance. Monolayers exposed to 2 μM A23187 for 1 hr were stained with fluorescein conjugated phalloidin, revealing that neighboring cells did not part one from another and that A23187 did not have a detectable effect on distribution of F-actin in the perijunctional actomyosin ring. The results indicate that elevation in intracellular Ca^{2+} decreases tight junction resistance in the T_{84} monolayer, acting through protein kinase C by a mechanism which does not involve visible changes in the perijunctional actomyosin ring.

Key words: Paracellular pathway — Intracellular free Ca^{2+} — Tight junctions — Protein kinase C

Introduction

There are two routes by which a solute can cross an epithelium: (i) the transcellular route, consisting of two membranes (the apical and basolateral) in series and (ii)

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the paracellular route, for which the intercellular tight junction is the rate-limiting barrier. While the transcellular pathway is classically viewed as the major route by which absorption of nutrients and Na^+ occurs, regulation of the passive permeation characteristics of the tight junction and therefore the paracellular pathway can substantially contribute to salt and nutrient absorption [1, 2, 31]. Similarly, transcellular electrogenic Cl^- secretion is coupled to paracellular passive secondary movement of Na^+ and water, although there is no evidence that the paracellular component of this response is regulated [18].

While it is known that a number of intracellular mediators (cAMP, cGMP, Ca^{2+}, calmodulin, diacylglycerol, phosphatidylinositol metabolites, and G-proteins) are involved in the regulation of intestinal absorption and secretion, the intracellular biochemical events that control structural and functional alterations in alimentary tract tight junctions are not well understood. Increasing concentrations of second messengers in intestinal epithelial cells have not produced consistent results. In *Necturus* gallbladder, cAMP decreased tight junctional ionic permeability in association with an increase in tight junction strand number and junctional depth [10]. An analogous effect of cAMP was observed in goldfish and flounder intestine [6]. Exposure of *Necturus* gallbladder to Ca^{2+}-ionophore increased transepithelial resistance, number of tight junctional strands, and junctional depth [30]. In contrast, Ca^{2+} ionophore decreased tight junctional resistance in rat liver [15]. Lowering of intracellular pH in canine gastric chief cell monolayers increased transepithelial resistance [25]. Thus, there is no consistent pattern in second messenger regulation of gastrointestinal tight junction resistance.

In this study, we discovered that elevation in intracellular Ca^{2+} ($[Ca^{2+}]_i$) decreases tight junction resistance in cultured T_{84} monolayers, and that the mechanism of this Ca^{2+} effect is mediated by protein kinase C.

Materials and Methods

Cell Culture

T_{84} cells obtained from the American Type Culture Collection (Rockville, MD) were grown as monolayers in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 25 mM $NaHCO_3$, 13.4 mM Na-Hepes, 90 μg/ml streptomycin, 90 U/ml penicillin, 5% newborn calf serum, 5% fetal calf serum. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Medium was changed three times per week and cells were used between passage 53–69. Cells were grown in plastic tissue culture flasks (Corning Glass Works, Corning, NY) until subconfluent, then suspended by incubation with 0.025% trypsin/0.265 mM EDTA solution and plated on rat tail collagen-coated polycarbonate filters (Nucleapore Corporation, Pleasanton, CA) as described [16]. Monolayers were studied 7–14 days following confluency.

Ussing Chamber Technique to Measure Transepithelial Solute Fluxes and Resistance

T_{84} monolayers were mounted in modified Ussing chambers (0.8 cm^2 exposed surface area). Serosal and mucosal reservoirs (4.5 ml each) contained oxygenated Ringer's-HCO_3 solution consisting of (in mM): 115 NaCl, 25 $NaHCO_3$, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$, and 1.2 $MgCl_2$, pH 7.4 at 37°C. Chambers were equipped with calomel electrodes and Ag/AgCl electrodes connected to the chambers via agar bridges for measurement of potential difference (PD) and short-circuit current (Isc), respectively by an automatic voltage clamp, DVC 1000 (WPI, Sarasota, FL). Unidirectional Na^+ and mannitol serosal-to-mucosal fluxes were simultaneously measured using $^{22}Na^+$ and [^{3}H]-mannitol. Both the mucosal and serosal surfaces of the T_{84} monolayer were bathed in the Ringer's-HCO_3 solution containing 5 mM mannitol. 5 μCi [^{3}H]-mannitol and 2 μCi $^{22}Na^+$ were added to the serosal side of the monolayer approximately 20 min after the monolayer was mounted in the Ussing chamber. One ml aliquots were taken from the mucosal bath starting at 30 min after the addition of isotopes and at 20, 25, 45, 65 and 85 min following the initial sample. Two μM A23187 were added to both the mucosal and serosal solutions immediately after the second sample. Each sample was replaced by one ml Ringer's-HCO_3 solution without isotopes. Two samples of 100 ml were taken from the serosal solution, one before the first sample and one after the last sample from the mucosal solution, for measuring the specific activities of [^{3}H]mannitol and $^{22}Na^+$. The activities of the two isotopes were separated through sequential counting in gamma and beta counters. The transepithelial resistance was measured by either the deflection in voltage in response to a current pulse of 100 mA or the change in current which resulted from voltage clamping at ±20 mV, both of which gave equivalent resistances.

Measurement of Intracellular Free Ca^{2+}

For free intracellular Ca^{2+} measurement by fluorometry of whole menolayers, T94 cells were seeded onto glass coverslips coated with rat tail collagen. The coverslips were glued to plastic supports with silicone rubber adhesive (General Electric, Waterford, NY) and studied 7–15 days after seeding. Cells were loaded with Fura-2 by incubation of the cells at 23°C for 60 min in ''NaCl Ringer solution'' containing (in mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 8 NaOH, 20 HEPES, pH 7.40, plus 5 μM Fura-2-AM followed by a 30-min incubation at 37°C. After washing three times with NaCl Ringer solution, the cells on the coverslips were mounted at 45° in a glass cuvette. Fluorescence was measured in an SLM spectrofluorometer (model SPF 500C, SLM, Urbana, IL) equipped with a cuvette stirrer and kept at 37°C. Wavelengths were computer controlled at excitations 340 ± 1 nm and 380 ± 1 nm, and emission was monitored at 505 ± 20 nm. Autofluorescence, determined from a filter seeded with cells but not loaded with dye, was subtracted automatically from the experimental reading. R_o was calculated at 3-sec intervals. After each experiment R_{max} was determined for each coverslip by the addition of 10 μM ionomycin in the presence of 2 mM Ca^{2+}, and R_{min} was determined by addition of 8.3 mM EGTA. Calculation of $[Ca^{2+}]_i$ was made as described previously [10]. In some condition BAPTA-AM (30 μM) was added 45 min before the Fura-2 and/or 4-Br-A23187 (2 μM) was added with the Fura-2.

Histology and Fluorescence Localization

Visualization of T_{84} cells by phase, DIC, and fluorescence localization of F-actin was carried out at the light microscopy level, as previously reported in detail [13].

Statistical Analysis

Transepithelial resistance was normalized with respect to the initial resistance for each monolayer. Statistical analysis was carried out using Student's *t* test for paired or unpaired variates.

Materials

A23187, H-7, W13, sphingosine, phorbol 12-myristate 13-acetate (PMA), 4a-phorbol, nordihydroguaiaretic acid (NDGA), mepacrine, and indomethacin were purchased from Sigma Chemical (St. Louis, MO), SKF 525A was from Smith, Kline, and Beckman (King of Prussia, PA), and $^{22}Na^{+}$ and [^{3}H]mannitol were from New England Nuclear (Boston, MA). BAPTA-AM, 4-Br-A23187, and FURA-2-AM were from Molecular Probes (Junction City, OR).

Results

Effect of Ca^{2+} Ionophore A23187 on T_{84} Monolayer Resistance

Ca^{2+} ionophore A23187 exposure caused a time- and concentration-dependent decrease in T_{84} monolayer resistance (Fig. 1*A*), which was detectable within 10 min and which increased over time. Control monolayer resistance decreased only very gradually, reaching 90% of the initial resistance at 60 min (Fig. 1), 70% at 120 min and 50% at 180 min (Fig. 7*A*). Most studies of the Ca^{2+} ionophore effect was performed over 60 min.

The A23187 concentration range of 10^{-7} to 10^{-5} M was studied as it encompassed the threshold and maximal responses in short-circuit current, previously reported in T_{84} cells (Fig. 1*A*) [23]. During the 60 min after A23187 addition, T_{84} cells exposed to 0.1 μM A23187 did not differ significantly in resistance from control; 1 μM and 2 μM A23187 caused an equivalent and gradual decrease in resistance to 45% of the baseline values; while 10 μM A23187 caused a much faster decrease in monolayer resistance which reached 17% of the baseline values in 60 min. As shown in Fig. 7*A*, 2 μM A23187 caused a decrease in monolayer resistance to approximately 20% of baseline values 120–180 min after ionophore was added.

The concentration dependence of the A23187 effect was studied on resistance 60 min after exposure. As shown in Fig. 1*B*, A23187 studied between 10^{-7} to 10^{-5} M caused a concentration dependent reduction in T_{84} monolayer resistance with a maximal effect being reached at 4 μM.

The reversibility of the A23187 effect on the monolayer resistance was examined by exposing the monolayers to A23187 (2 μM) for 1 hr, then washing with a Ringer's-HCO_3 solution containing 1% BSA. Figure 2 shows that the decrease in monolayer resistance caused by A23187 was partially reversed as soon as 5 min after washing; while similarly washed control monolayers did not show an increase in their resistance.

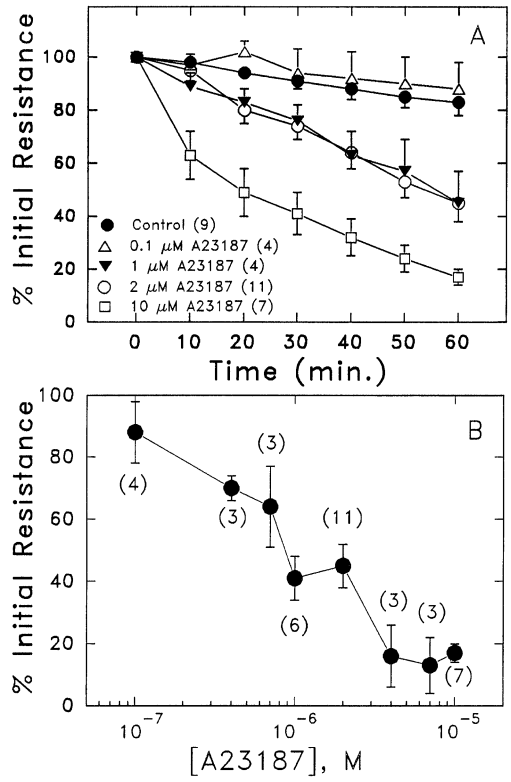

Fig. 1. (*A*) Time and concentration dependence of A23187 effect on T_{84} resistance. Time course of transepithelial resistance (expressed as % initial resistance) of T_{84} monolayers exposed to 0, 0.1, 1, 2, and 10 μM A23187. The 20 to 60 min values of 1 μM and 2 μM A23187 exposed monolayers are significantly different from those of control and 10 μM A23187 values ($P < .01$). All values of 10 μM A23187 exposed monolayers are significantly less than control. The final concentrations of ethanol used as solvent for A23187 were 0.05%, 0.l%, and 0.5% for 1 μM, 2 μM, and 10 μM A23187, respectively. As shown in Fig. 7*A*, 1% EtOH did not significantly alter resistance after up to 180 min of exposure. Numbers in parentheses are the numbers of monolayers studied. (*B*) A graded dose-response to A23187 between 0.1 μM and 10.0 μM. Values were taken at 60 min after exposure to A23187. The results show that the effect of A23187 on T_{84} monolayer resistance is gradually increased and reaches maximum at 4 μM. Numbers in parentheses are the number of monolayers studied.

Dual Na^{+}/Mannitol Serosal-to-Mucosal Flux Analysis in A23187-treated T_{84} Monolayers

Using dual Na^{+}-mannitol flux analysis, Madara et al. [22] showed that differences in baseline monolayer resistance of T_{84} cells represented variations in tight junction resistance and that a cytochalasin D-induced decrease in T_{84} monolayer resistance also represented decreases in tight junction resistance. This is also true for the effects of Ca^{2+} ionophore A23187 in T_{84} monolayers.

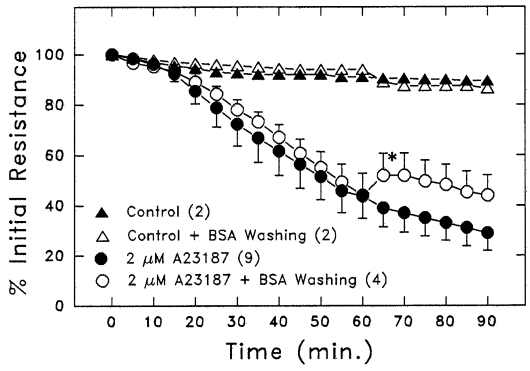

Fig. 2. Reversibility of the A23187-induced decrease in tight junction resistance of T_{84} monolayers. Two μM A23187 were added to the mucosal plus serosal surfaces at 0 time. At 60 min, the control and 2 μM A23187 exposed monolayers were washed 3 times rapidly on both mucosal plus serosal surfaces with a Ringer's-HCO_3 solution without A23187 but containing 1% BSA. The resistance in control monolayers was not significantly altered but the decline of tight junction resistance caused by A23187 was partially reversed by washing. *The mean value 5 min after BSA wash was significantly higher ($P < 0.05$) than the prewash value by paired Student's *t*-test. Numbers in parentheses are the number of monolayers studied.

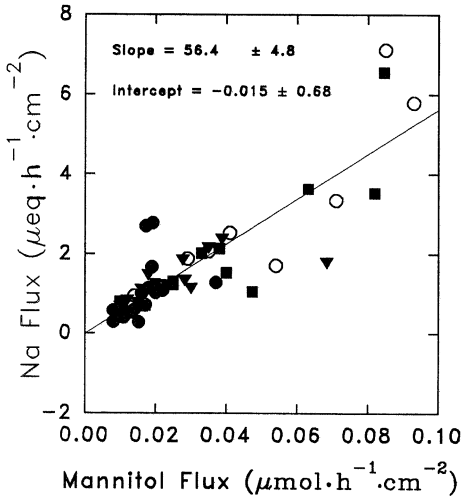

Fig. 3. Dual serosal-to-mucosal $^{22}Na^+$ and [^{3}H]mannitol flux relationship in T_{84} monolayers exposed to Ca^{2+} ionophore A23187. Serosal-to-mucosal unidirectional fluxes of Na^+ and D-mannitol were measured simultaneously using $^{22}Na^+$ and [^{3}H]mannitol across T_{84} monolayers treated with 2 μM A23187. A control 20-min flux was measured 30–50 min after isotopes were added to the serosal solution, then 2 μM A23187 were added to the mucosal plus serosal surfaces at 50 min following isotope addition and three further 20 min fluxes were determined. Individual data points are shown: closed circles are fluxes measured before A23187 addition; closed triangles, closed squares, and open circles are the first, second, and third 20-min fluxes after A23187 addition, respectively. The line was drawn by linear regression/least squares analysis.

Dual Na^+-mannitol flux analysis also showed that the Ca^{2+} ionophore-induced decrease in T_{84} monolayer resistance represented increased tight junction permeability. A23187 increased serosal-to-mucosal fluxes of both Na^+ and mannitol (1.05 ± 0.21 μeq/hr/cm^2 for Na^+ and 0.017 ± 0.002 μmol/hr/cm^2 for mannitol before exposure to A23187 and 2.90 ± 0.73 μeq/hr/cm^2 for Na^+ and 0.049 ± 0.010 μmol/hr/cm^2 for mannitol 60 min after exposure to A23187, $P < 0.01$ for both Na^+ and mannitol fluxes). In addition, as shown in Fig. 3, a linear relationship exists between unidirectional serosal-to-mucosal Na^+ and mannitol fluxes in T_{84} monolayers treated with 2 μM A23187. The slope of the Na^+/mannitol relationship (56.4) is in good agreement with the theoretical value for free diffusion (54), (note that the control points and the points in the presence of A23187 fall on the same line), indicating that resistance variations across these monolayers are solely due to variations in tight junction permeability [5].

BAPTA Loading Inhibits the A23187-induced Decrease in T_{84} Resistance

The Ca^{2+} ionophore A23187-induced decrease in the T_{84} monolayer resistance was consistent with intracellular or extracellular Ca^{2+} being involved in regulation of tight junction resistance. The $[Ca^{2+}]_i$ chelator BAPTA was used to examine the role of intracellular Ca^{2+} in the A23187 action on the T_{84} monolayers resistance. The monolayers were incubated with 30 μM BAPTA-AM for 45 min to load the membrane-permeant acetoxymethyltetraester form of the compound into the intact cells prior to the addition of 2 μM A23187. Figure 4 shows that 105 min after exposure to BAPTA-AM (45 min loading plus 60 min of study) there was a slight decrease in T_{84} monolayer resistance. When A23187 was added to BAPTA loaded cells, the ionophore-induced decrease in resistance was inhibited. By 25 min after addition of A23187, the decrease in the monolayer resistance was significantly reduced by the presence of 30 μM BAPTA.

$[Ca^{2+}]_i$ in T_{84} cells with and without preincubation with 30 μM BAPTA-AM in the presence and absence of 2 mM A23187 were measured using FURA-2-AM with a fluorometer. The results are shown in the Table. BAPTA-AM loading significantly decreased the $[Ca^{2+}]_i$ in control and A23187-exposed T_{84} cells. The BAPTA-induced decrease in $[Ca^{2+}]_i$ also reduces the T_{84} monolayer resistance (Fig. 4).

Ca^{2+} Ionophore-induced Decrease in T_{84} Monolayer Resistance is Mediated by Protein Kinase C Dependent Mechanism

H-7 and W-13

H-7 (60 μM) and W13 (45 μM), inhibitors of Ca^{2+}-dependent protein kinase C and calmodulin kinase II,

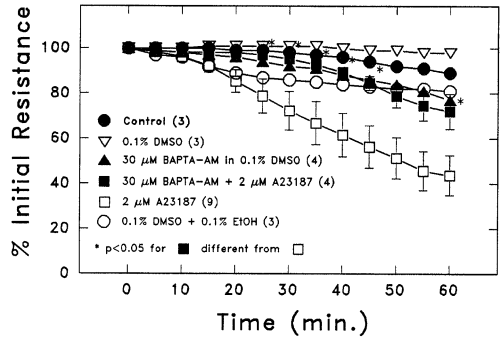

Fig. 4. Effect of the intracellular Ca^{2+} chelator, BAPTA, on the A23187-induced tight junction resistance. Preincubation of T_{84} monolayers with 30 μM BAPTA-AM for 45 min caused a small decrease in resistance by itself. BAPTA significantly inhibited the decrease in tight junction resistance induced by 2 μM A23187. The solvent for BAPTA alone (0.1% DMSO) did not affect the tight junction resistance nor did the combination of solvents used for BAPTA plus A23187 (0.1% DMSO plus 0.1% EtOH). Numbers in parentheses are the number of monolayers studied.

Table. $[Ca^{2+}]_i$ concentration in control and BAPTA-AM-pretreated T_{84} monolayers

	Control (6)	A23187 (5)	BAPTA-AM (4)	BAPTA-AM + A23187 (5)
$[Ca^{2+}]_i$ (nM)	194 ± 35	278 ± 38	100 ± 12[a]	136 ± 17[b]

T_{84} monolayers grown on glass coverslips were preincubated in NaCl Ringer solution with or without 30 μM BAPTA-AM for 45 min and followed by exposure to 5 μM FURA-2-AM with or without 2 μM 4-Br-A23187 for 45 to 60 min. $[Ca^{2+}]_i$ was then measured with a fluorometer. Results are mean ± SEM. Numbers in parentheses are the number of monolayers studied. [a]$P < 0.025$ compared to untreated control without BAPTA-AM; [b]$P < 0.02$ compared to A23187 without BAPTA-AM.

respectively, were studied for their effect on basal T_{84} monolayer resistance and on the A23187-induced decrease in resistance. H-7 (Fig. 5*A*) and W13 (Fig. 5*B*) alone did not significantly decrease the T_{84} monolayer resistance compared to the control. In fact, W13 caused a slight but insignificant increase in resistance in otherwise untreated T_{84} monolayers.

As shown in Fig. 5*A*, H-7 totally inhibited the decrease in the monolayer resistance induced by 2 μM A23187. In contrast, W13 did not inhibit the A23187-induced decrease in resistance (Fig. 5*B*).

Sphingosine

To support the H-7 studies implicating protein kinase C in mediating the decrease in the tight junction resistance caused by A23187, sphingosine, another inhibitor of protein kinase C, was used (Fig. 6). 50 μM sphingosine was

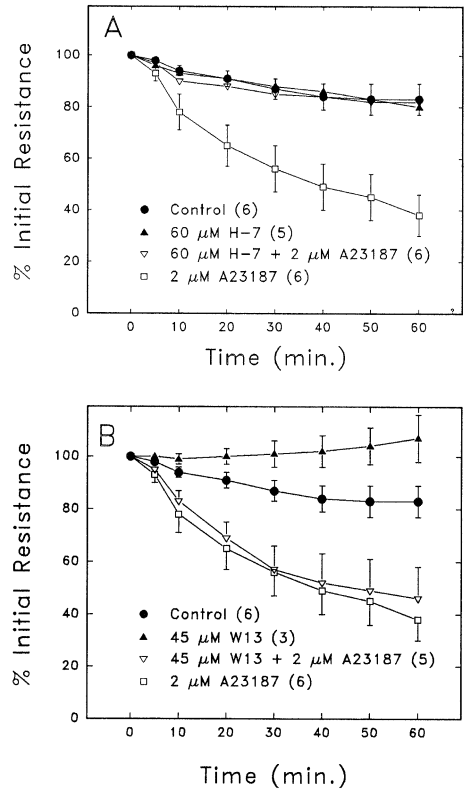

Fig. 5. (*A*) Effect of the protein kinase C inhibitor, H-7, on the decrease in tight junction resistance caused by 2 μM A23187. The T_{84} monolayers were preincubated in 60 μM H-7 for 15 min prior to exposure to 2 μM A23187. H-7 totally abolished the A23187 effect on tight junction resistance. Numbers in parentheses are the number of monolayers studied. (*B*) Effect of the calmodulin kinase II inhibitor, W13, on the decrease in tight junction resistance caused by 2 μM A23187. The T_{84} monolayers were preincubated in 45 μM W13 for 15 min prior to exposure to 2 μM A23187. W13 caused a slight but insignificant increase in baseline T_{84} monolayer resistance; but W13 had no effect on the A23187-induced decrease in tight junction resistance. Numbers in parentheses are the number of monolayers studied.

added to the bathing solutions 30 min before adding 2 μM A23187. Sphingosine did not significantly alter basal T_{84} resistance but did inhibit the decrease in resistance caused by A23187; however, the effect was delayed compared to H-7, only becoming significant starting 50 min after the addition of A23187.

Effect of Phorbol Esters on T_{84} Monolayer Resistance

Based on the above inhibitor studies, we hypothesized that the protein kinase C stimulator PMA would cause a similar effect to that of A23187 on T_{84} tight junction resistance. However, PMA induced a complex pattern of resistance changes (Fig. 7*A*). After a 10-min delay, resistance increased, returned to the initial value at 80 min

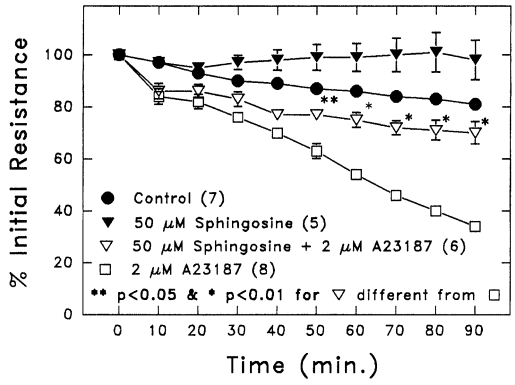

Fig. 6. Effect of the PKC inhibitor, sphingosine, on the decrease in T_{84} tight junction resistance caused by 2 μM A23187. Preincubation with 50 μM sphingosine on the mucosal plus serosal surfaces for 30 min before A23187 exposure significantly decreased the tight junction resistance effect of A23187. The effect was significant starting at 50 min of A23187 exposure. A23187 in sphingosine pretreated monolayers caused a significantly smaller decrease in resistance than in monolayer treated with A23187 alone: $^{a}P < 0.05$ and $^{b}P < 0.01$. Numbers in parentheses are the number of monolayers studied.

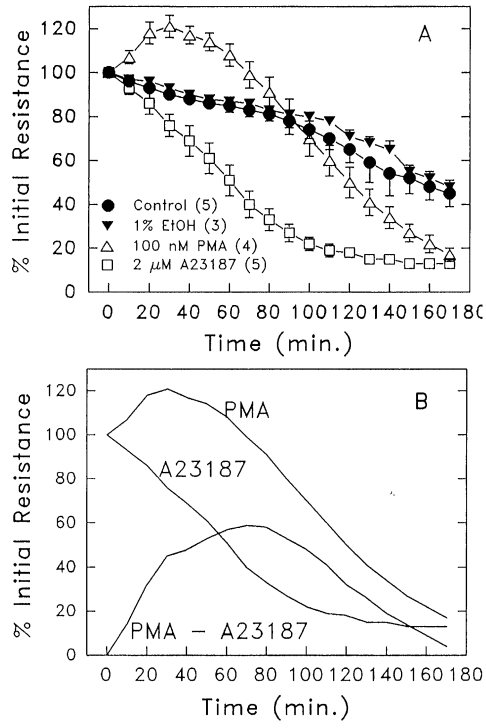

Fig. 7. (*A*) Effect of the PKC activator, 4β-PMA (100 nM), on tight junction resistance of T_{84} monolayers. 4β-PMA were added to the mucosal plus serosal surfaces at time 0. The effect of PMA was different from that of 2 μM A23187; there was an increase in resistance followed by a decrease similar to that caused by A23187, but which was delayed for 1 hr. Resistance in the monolayer exposed to 1% ethanol (maximal vehicle control) was not significantly different from untreated control monolayers. Final concentration of EtOH in the 4β-phorbol solutions was 0.1%. Numbers in parentheses are the number of monolayers studied. (*B*) Dual effect of 4β-PMA on tight junction resistance of T_{84} monolayers. The PMA and A23187 curves are the same ones in *A*. The PMA curve can be split into two components: the first component is the A23187 curve and the second component is the difference between these two curves shown as the PMA-A23187 curve. The second component of PMA curve shows a PMA-stimulated increase in resistance that lasts about two hr.

and then decreased to a level close to that caused by A23187. Thus, there was a 1-hr delay in the decrease of resistance compared with that due to the Ca^{2+} ionophore. The parallelism in the decay of resistance between the PMA and A23187 curves suggests that PMA curve contains a component similar to that of A23187. A second component of the PMA curve was obtained by subtracting the PMA and A23187 curves; this indicates a PMA-stimulated increase in resistance that lasts about two hours and is shown in Fig. 7*B*. In contrast to the effect of the β-phorbol (100 nM), PMA, an inactive form of phorbol ester, α-phorbol (100 nM), caused no effect on resistance when exposed to T_{84} monolayers for up to 120 min (*data not shown*). Also 100 nM, 1,2-dioctanoyl-sn-glycerol (DiC_8) when added every 15 min caused a change in resistance similar to that caused by PMA, causing an increase in resistance for 60 min and then producing a decrease in resistance (*data not shown*).

Effect of a Phospholipase Inhibitor (Mepacrine) and Inhibitors of Arachidonic Acid Metabolism on the Changes in T_{84} Resistance Caused by A23187

To test whether a phospholipase or arachidonic acid metabolites were also involved in the A23187-induced decrease in tight junction resistance, the effects of mepacrine (a general phospholipase inhibitor), indomethacin (an inhibitor of the cyclooxygenase pathway of arachidonic acid metabolism), NDGA (an inhibitor of the lipoxygenase pathway), and SKF 525A (an inhibitor of the epoxygenase pathway) were studied on T_{84} resistance in the presence and absence of 2 mM A23187. Mepacrine added to the mucosal plus serosal surfaces caused a slight but significant increase in basal T_{84} resistance, which returned to control values in 80 min while SKF 525A, indomethacin, and NDGA did not have significant effects on resistance (*data not shown*). In contrast, none of these agents added with A23187 caused a substantial reduction of the decrease in tight junction resistance caused by 2 μM A23187 (*data not shown*).

Effect of A23187 on Morphology of T_{84} Monolayers

To examine whether A23187 elicited changes in tight junction permeability by producing detectable alterations in cell-cell contact or in the perijunctional actomyosin ring, phase, DIC, and fluorescence imaging were per-

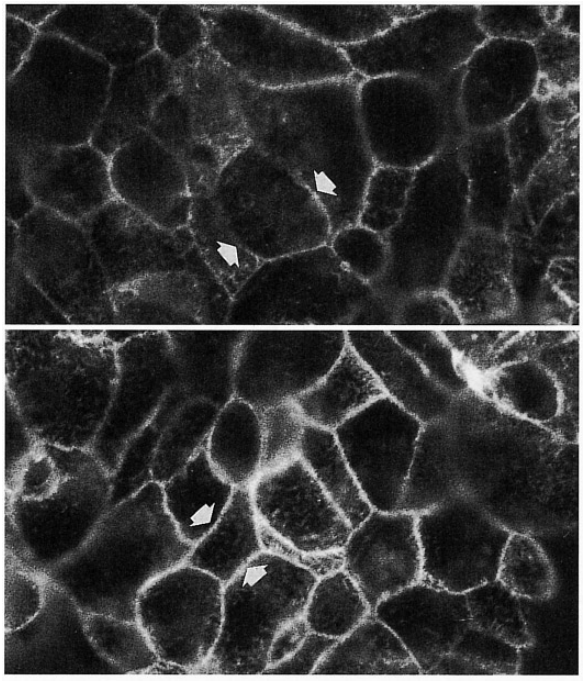

Fig. 8. En face fluorescence localization of perijunctional F-actin. In T_{84} monolayers exposed (bottom) or unexposed (top) to 2 μM A23187 for 60 min, the perijunctional rings of adjacent cells (arrowheads) appear as fused bands indicating maintenance of close cell-cell apposition. Moreover, the appearance of F-actin in the ring is comparable between control and A23187 exposed monolayers (magnification, 1500X).

formed on monolayers exposed to a half-maximal concentration of ionophore (2 μM) for 30–60 min. As shown in Fig. 8, fluorescence localization of F-actin in the plane of the apical perijunctional actomyosin ring showed that ring phenotype was unaffected under these conditions. Also shown in this figure, and confirmed by DIC microscopy, is that individual cells remained tightly adjacent to their neighbors (i.e., actomyosin rings of neighboring cells appear as a single fluorescence band).

Discussion

The effect of Ca^{2+} ionophore to decrease epithelial tight junction resistance as demonstrated in this study was previously observed in monolayers of Caco-2 and MDCK cells [24, 34]. However, the mechanism of this effect was not studied.

The results of this study suggest that elevation in intracellular Ca^{2+} increases T_{84} monolayer permeability through effects on intracellular tight junctions. However, unlike the initially described effects of depletion of extracellular Ca^{2+} on intestinal tight junctions [8], the effects of raising intracellular Ca^{2+} are not related to gross disassembly of cell-cell contacts. Rather, cells remain in contact under conditions in which elevated intracellular Ca^{2+} enhances junction permeability. Similarly, no detectable alterations in F-actin distribution in the perijunctional ring occurred under these latter conditions. In previously reported examples of enhanced T_{84}, tight junction permeability elicited with cytochalasin D [19], *C. difficile* toxin A [16], and gamma interferon [21], cell-cell continuity was also maintained. However, in these studies, redistribution (often subtle) of perijunctional F-actin was detected. Thus, it is likely that the effects of elevated intracellular Ca^{2+} on tight junction permeability either are accompanied by perijunctional cytoskeletal effects too subtle to be detected by the methods employed (for example enhanced actomyosin ring tension without a change in morphology) or are attained through different mechanisms than are the effects of the other perturbations listed above.

This effect of elevation in intracellular Ca^{2+} to lower tight junction resistance appears to be mediated by protein kinase C. Not only is the effect of elevating Ca^{2+} ionophore blocked by the protein kinase C inhibitors H-7 and sphingosine and not by the calmodulin kinase II inhibitor W13, but also the effects of Ca^{2+} were partially duplicated by exposure to the β-phorbol ester, PMA and DiC_8, while the inactive α-phorbol ester was without effect. Although PMA decreased resistance that merged with the A23187 curve in about three hours, the time course is different from that of A23187 (Fig. 7*A*). However the close parallelism in the decay of resistance between the PMA and A23187 curves suggests that the PMA curve contains a component similar to that of A23187. The second component obtained as the difference between the PMA curve and A23187 curve shows a PMA-stimulated increase in resistance that lasts about 2 hr. The mechanism of this component is not known.

Previous studies have suggested a role for protein kinase C in acute regulation of epithelial tight junction permeability. Most of these studies have evaluated only the effects of the acute addition of phorbol esters. The effect of phorbol esters varied among epithelial types. In most, phorbol esters increased tight junction permeability including in LLC-PK_1B, MDCK [11], LLC-PK_1 [27], and hepatocyte couplets [28]; while in others, it decreased tight junction permeability (LLC-PK_1A [11]) or did not alter tight junction permeability (intact rat liver [32]). While studies on the mechanism were pointedly lacking, in one study (in LLC-PK_1) diacylglycerols duplicated the effect of phorbol esters on tight junction permeability but the reversibility of the change was different [26]. The diacylglycerol effect was reversible but not the effect of phorbol esters. In addition, the phorbol ester effects on tight junction permeability in MDCK, the distal renal tubular cell line [29] and in LLC-PK_1, a proximal tubule cell model [27], did not require protein synthesis and was not affected by agents which interfere with cytoskeletal function, including cytochalasin B and

D, colchicine and vinblastine [29]. It was suggested that tight junction resistance (or paracellular permeability) is regulated by the activity of enterocyte PKC in a study of transepithelial resistance response of Caco-2 monolayers to the phorbol ester PMA [35]. Thus, protein kinase C in both intestinal and kidney cell lines regulates epithelial tight junction resistance by decreasing it. We have no data concerning the events involved in the early PMA-induced increase in resistance. However, the biphasic time course in resistance response to phorbol ester 12-0-tetradecanoylphorbol 13-acetate (TPA) was consistent with downregulation of PKC as observed by Hecht et al. [14]. Please note however, PMA reaches all cell membranes and there is no reason to think its effects would be limited to the tight junction.

Emphasis in the current studies was on the effects of elevation in Ca^{2+} on tight junction resistance for up to 1 hr. More prolonged elevation of protein kinase C, as caused by exposure to phorbol esters in T_{84} cells, alters transport in an unusual way; not only is there a down-regulation of C kinase activity but there is a prolonged activation of phospholipase C, which leads to a prolonged elevation in basal IP_3, and an eventual emptying of intracellular calcium stores [33]. This complex response precludes mechanisms being inferred for the effect of PMA on T_{84} resistance shown in Fig. 7.

The mechanism by which intracellular calcium acting by protein kinase C affects tight junctions is not known. One possibility is that protein kinase C may translocate to its site of action at the tight junction or to the perijunctional area, where it could enzymatically influence the proteins which regulate tight junctional function, for instance for ZO-1 protein [36] or actin. We also considered that protein kinase C could potentially act via a phospholipase or via a change in arachidonic acid production. Recently, it has been demonstrated that changes in intracellular Ca^{2+} alter active NaCl absorption in ileal villus Na^+ absorbing cells at least partially by affecting a membrane bound phospholipase [7, 9]. This does not appear to be the case in regulation of tight junction resistance in T_{84} cells by elevation in intracellular Ca^{2+}, since a general phospholipase inhibitor, as well as inhibitors of all three major pathways of arachidonic acid metabolism, cyclooxygenase, lipoxygenase, and epoxygenase, failed to alter the ionophore-induced changes in T_{84} tight junction resistance.

Not explained in detail is that lowering intracellular free Ca^{2+} also regulates T_{84} monolayer resistance, lowering it. This was concluded from the BAPTA experiments which lowered intracellular Ca^{2+} and were associated with a decrease in tight junction resistance (Fig. 4). Multiple examples of Ca^{2+} acting via calmodulin have been associated with a concentration dependence in which both increases and decreases from the maximal effective concentration lessen the effect [12]. Multiple mechanisms have accounted for this observation. No exploration of the mechanism of the effects of the decreased Ca^{2+} on tight junction resistance has been pursued as yet.

It is now clear that intestinal epithelial tight junctions are regulated under physiologic conditions [1, 17, 20] and in models of several pathologic conditions [6, 21]. However, it is less clear what intracellular signaling cascades are responsible for such regulation. Our results suggest that, as modeled by T_{84} cells, Ca^{2+}-mediated kinase activation can modulate tight junction permeability; and it appears that these effects are dependent on protein kinase C rather than on a calmodulin-dependent kinase II. In MDCK monolayers, Citi [4] showed that protein kinases are involved in regulating the decrease in transepithelial resistance caused by lowering the extracellular calcium concentration and Balda et al. [3] suggested that both PKC and calmodulin are involved in the development of transepithelial electrical resistance during the making and sealing of a tight junction. We speculate that similar events may be involved in regulating tight junction permeability in natural epithelia during normal digestion and in diarrheal diseases. This would alter the contribution of solvent drag and thus change the amount of absorption and secretion of water and electrolytes, as well as affect the barrier function of the gut. Thus, the changes in tight junction permeability described here may be involved in regulating both normal water and electrolyte transport as well as creating some of the pathobiological events seen in diarrheal diseases.

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