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Assembly and Sealing of Tight Junctions: Possible Participation of G-proteins, Phospholipase C, Protein Kinase C and Calmodulin

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Summary. The making and sealing of a tight junction (TJ) requires cell-cell contacts and Ca^{2+}, and can be gauged through the development of transepithelial electrical resistance (TER) and the accumulation of ZO-1 peptide at the cell borders. We observe that pertussis toxin increases TER, while AlF_3 and carbamil choline (carbachol) inhibit it, and 5-guanylylimidodiphosphate (GTPΓs) blocks the development of a cell border pattern of ZO-1, suggesting that G-proteins are involved. Phospholipase C (PLC) and protein kinase C (PKC) probably participate in these processes since (i) activation of PLC by thyrotropin-1 releasing hormone increases TER, and its inhibition by neomycin blocks the development of this resistance; (ii) 1,2-dioctanoylglycerol, an activator of PKC, stimulates TER development, while polymyxin B and 1-(5-isoquinoline sulfonyl)-2-methyl-piperazine dihydrochloride (H7), which inhibit this enzyme, abolish TER. Addition of 3-isobutyl-1-methyl-xanthine, dB-cAMP or forskolin do not enhance the value of TER, but have just the opposite effect. Trifluoperazine and calmidazoline inhibit TER development, suggesting that calmodulin (CaM) also plays a role in junction formation. These results indicate that junction formation may be controlled by a network of reactions where G-proteins, phospholipase C, adenylate cyclase, protein kinase C and CaM are involved.

Key Words epithelia · tight junctions · G-proteins · Ca^{2+} · MDCK phospholipase C · protein kinase C · calmodulin · exocytic fusion

Introduction

Cells from the Madin-Darby canine kidney (MDCK) epithelial line plated at confluence, form monolayers that establish tight junctions (TJs) through a process that requires cell-cell contacts and Ca^{2+}. This process can be followed through the development of a transepithelial electrical resistance (TER) that reaches a maximum in 12–15 hr (Cereijido et al., 1978*a*,*b*). Yet, if shortly after plating monolayers are transferred to media without Ca^{2+}, they do synthesize junctional components, but these seem to be retained in an intracellular compartment placed between the Golgi apparatus and the surface membrane, and TER remains negligible (González-Mariscal, Chavez de Ramirez & Cereijido, 1985). Addition of Ca^{2+} to these monolayers (*Ca switch*) provokes (i) an exocytic fusion that increases the surface membrane by 22%; (ii) the appearance of junctional strands in freeze-fracture replicas; (iii) a blockade of the diffusion of ruthenium red through the intercellular space; and (iv) a faster development of TER (4–6 hr instead of 12–15) (González-Mariscal et al., 1985, 1990).

Ca triggering of junction formation requires the presence of this ion on the extracellular side, as the blockade of its penetration by La^{3+} fails to suppress junction formation (González-Mariscal et al., 1990). This indicates that the cell membrane must have a mechanism for transducing the Ca signal to intracellular effectors.

Moreover, while all epithelial cells in a given organism are exposed to contacts with their neighbors as well as to the same Ca^{2+} concentration in the interstitium, the degree of sealing of their TJs varies over several orders of magnitude, from the proximal tubule of the kidney (8–10 $\Omega \cdot cm^2$) to the mucosa of the urinary bladder (several thousands Ω/cm^2). Therefore, besides cell contacts and Ca^{2+}, there must be some additional factors or mechanisms that affect the degree of sealing.

Therefore, in the present work we use the Ca switch to investigate some mechanisms that may be intercalated between Ca^{2+} triggering and junctional sealing.

Materials and Methods

Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34). Cells were grown at 36.5°C

in disposable plastic bottles (Costar 3150, Cambridge, MA) with an air-5% CO_2 atmosphere (VIP CO_2 incubator 417, Lab. Line Instruments, New Brunswick, NY) and 20 ml of Dulbecco's modified Eagle's basal medium (DMEM, Grand Island Biological -GIBCO- 430-1600, Grand Island, NY) with 100 U/ml of penicillin, 100 μg/ml of streptomycin (In vitro S.A., México D.F.), 0.08 U/ml of insulin (Eli Lilly, México D.F.), and 10% fetal bovine serum (Flow Laboratories, McLean, VA); this complete medium is referred to in the following text as normal calcium (NC) medium. Cells were harvested with trypsin-EDTA (In Vitro S.A., México, D.F.) and plated on nitrocellulose filters (HAWP 293-25; pore diameter: 0.45 μm, Millipore, Bedford, MA). Upon allowing 1 hr for cell attachment, medium was discarded, and monolayers were switched to fresh media.

Ca Switch

Once cells were left to attach for 1 hr as described above, the resulting monolayers were washed three times with PBS without Ca^{2+} (GIBCO 450-1300), and transferred to Minimal Essential Medium (GIBCO 410-1300) without Ca^{2+}, [determinations with a Ca^{2+}-sensitive electrode detects 1–4 μM Ca^{2+}; therefore, we refer to this medium as low calcium (LC)]. Twenty hours later the experimental protocol was started by changing the media to NC medium.

Transepithelial Electrical Resistance (TER)

The degree of sealing of the tight junctions was assessed by measuring the TER. After incubation under a given condition, the filter with the monolayer was mounted as a flat sheet between two Lucite chambers with an exposed area of 0.23 cm^2. Current was delivered via Ag/AgCl electrodes placed at 2.0 cm from the monolayer; the voltage deflection elicited was measured with a second set of electrodes placed at 1.0 mm from the membrane. Values of TER reported were obtained by subtracting the contribution of the filter and the bathing solution. A given monolayer was used only for a single determination and discarded to avoid leaks due to edge damage.

Abbreviations and Source of Chemicals

CTX: cholera toxin (from vibrio Cholera); GTPγs: 5-guanylylimidodiphosphate (sodium salt); IBMX: 3-isobutyl-1-methyl-xanthine; dB-cAMP: N^6,$O^{2'}$-dibutyryladenosine 3'-5'-cyclic monophosphate; forskolin; PMB: polymyxin B sulfate; TFPZ: trifluoperazine dihydrochloride; carbachol: carbamil choline; TRH: thyrotropin-releasing hormone and neomycin sulfate were obtained from Sigma Chemical (St. Louis, MO). H7: 1-(5-isoquinoline sulfonyl)-2-methyl-piperazine dihydrochloride; EGD: ethylene glycol dioctanoate; and diC8: 1,2-dioctanoylglycerol were from Molecular Probes (Eugene, OR). CMZ: calmidazoline from Janssen Pharmaceutical (Beerzen, Belgium). Aluminum Fluoride was obtained from Aldrich (Milwaukee, WI). PTX: pertussis toxin (islet activating protein) was purified from pertussis vaccine concentrates generously provided by the National Institute of Hygiene (México) by the method of Sekura et al. (1983). Other abbreviations are: MDCK: Madin Darby Canine Kidney cells; TJ: tight junction; TER: transepithelial electrical resistance; G_i: inhibitory G-protein; G_s: stimulatory G-protein; PIP_2: phosphatidyl 4,5-diphosphoinositol; IP_3: inositol 1,4,5-trisphosphate; PLC: phospholipase C; PKC: protein kinase C; DAG: diacylglycerol. AC: adenylate cyclase; cAMP: cyclic adenosine monophosphate; CaM: calmodulin.

Treatment with Drugs

To guarantee a proper access of the drugs to the cells during a Ca switch, all of them were first added during the last 30 min of incubation in LC. The monolayers were then switched to NC medium containing the same drug. Some chemicals were dissolved and kept at −20°C in stocks prepared in DMSO: forskolin (24.6 mM); and diC8 (25 mg/ml). CMZ was daily dissolved in DMSO. In all cases the final concentration of DMSO was ≤0.5%; a concentration unharmful to these monolayers. Other drugs were prepared as stocks in H_2O and kept frozen at −20°C: PTX (50 μg/ml), GTPγs (100 mM), EGD (100 mg/ml), and TRH (7 mM). The rest of the drugs used were prepared daily and directly dissolved in low calcium DMEM. For each drug a trypan extrusion test was run to ensure that it would not affect cell viability.

Results are expressed as mean ± experimental error (number of observations).

Determination of Pertussis Toxin Substrate

ADP-ribosylation of proteins in cell membranes was determined by measuring the ^{32}P-ADP-ribose incorporation catalyzed by pertussis toxin in vitro. MDCK cells incubated in the presence (10, 100, 1000 ng/ml) and absence of PTX for 5 hr were washed and suspended in ice-cold buffer consisting of 25 mM Tris HCl, pH 7,4; 1 mM EDTA; 1 mM dithiothreitol; and 10 μg/ml aprotinin. Cellular homogenates were prepared with an homogenizer (10 strokes) and pelleted by centrifugation at 100,000 × g for 30 min (Portilla, Morrissey & Morrison, 1988).

The pellet was resuspended in the same buffer, and the protein concentration was measured by the method of Lowry et al. (1953). An aliquot of 200 μg of protein was incubated in a final volume of 100 μl at 30°C for 60 min in buffer containing 250 mM potassium phosphate, pH 7.5; 5 mM $MgCl_2$; 2 mM ATP; 0.1 mM GTP; 20 mM thymidine; 10 mM arginine; 0.75 mM $NADP^+$; 10 μCi α-^{32}P − NAD and 10 μg of PTX. PTX was preactivated with 20 mM dithiothreitol at 37°C for 10 min. A control in the absence of toxin was run in parallel. The reaction was terminated with 1 ml of cold potassium phosphate buffer, 100 μl of 50% TCA and 100 μl of 0.15% deoxycholate. The protein was precipitated, collected by centrifugation, resuspended in 100 μl of urea/Laemmli, and 5% of β-mercaptoethanol was added before boiling for 5 min at 100°C (Kawai, Whitsel & Arinze, 1986; Hernández-Sotomayor et al., 1990). The protein was separated by molecular mass on a 10% polyacrylamide SDS-containing gel by the method of Laemmli (1970). The gel was then stained, destained, dried and autoradiographed.

ZO-1 Indirect Immunofluorescence

Glass coverslips containing confluent monolayers of MDCK cells were rinsed twice with PBS, fixed and permeabilized with −20°C methanol for 45 sec. Monolayers were washed with PBS, incubated with 3% fetal bovine serum in PBS for 30 min, and treated overnight at 4°C with ZO-1 monoclonal antibody (R26.4C8C9C8, Stevenson et al., 1986, 1988) diluted 1:50 in 1% fetal bovine

serum-PBS. Cells were washed twice with PBS, incubated 30 min with 3% fetal bovine serum in PBS, stained with rhodamine conjugated rabbit anti-rat antibody (Sigma) for 60 min and washed twice with PBS. The glass coverslips were then mounted in *p*-phenyldiamine-glycerol (1 : 9) and examined with a Leitz Orthoplan microscope (Leitz, Wetzlar, Germany).

Actin Indirect Fluorescence

Confluent monolayers of MDCK cells on glass coverslips were rinsed twice with PBS, fixed with 1% formaldehyde, washed twice with PBS, treated with −20°C acetone for 5 min and dried with air. Monolayers were incubated for 60 min with a rhodamine phalloidin solution (1 : 20 in PBS). Glass coverslips were washed twice with PBS and mounted in *p* − phenyldiamine-glycerol (1 : 9) and viewed in a Leitz Orthoplan microscope (Leitz, Weitzlar, Germany).

Results are expressed as mean ± SE (number of observations).

Results

To facilitate comparisons between the effects of the different substances tested, the value of TER reported was normalized as follows:

$$TER = TER_e\ (X_{all}/X_e)$$

where TER_e is the value of TER measured in a given experimental monolayer, X_e is the mean value of the control group processed simultaneously, and X_{all} is the mean value of TERs measured in all control groups. Unless otherwise stated, TER was measured before and 5 hr after switching the monolayers from 1–4 μM to 1.8 mM Ca^{2+}.

Figure 1 shows that before the Ca switch, monolayers have a TER of 21 ± 7 (21) $\Omega \cdot cm^2$ (first column); if they are not switched to Ca^{2+} 5 hr later their TER remains low at 11 ± 3 (15) (second column), but if they are switched they develop a TER of 280 ± 20 (182) (third column). PTX (14 ng $\cdot$ ml^{-1}), which is known to inhibit the action of certain G-proteins (Murayama & Ui, 1984; García-Sáinz, 1985), increases the level of TER recorded at the 5th hour by up to 511% ($P \leq 0.001$; 4th column). However, the action of pertussis toxin by itself cannot onset the sealing of TJs, because if the concentration of Ca^{2+} is kept at 1–4 μM, TER remains low (last column).

To ensure that at the concentration used PTX was acting on G-proteins in MDCK cells, we measured the degree of ADP-ribosylation of cell homogenates pretreated with the toxin. This was performed in the presence of [α-^{32}P]-NAD. Figure 2 shows that, in fact, PTX reduces in a dose-dependent manner the degree of labeling of a 41-kD protein. Densitometric

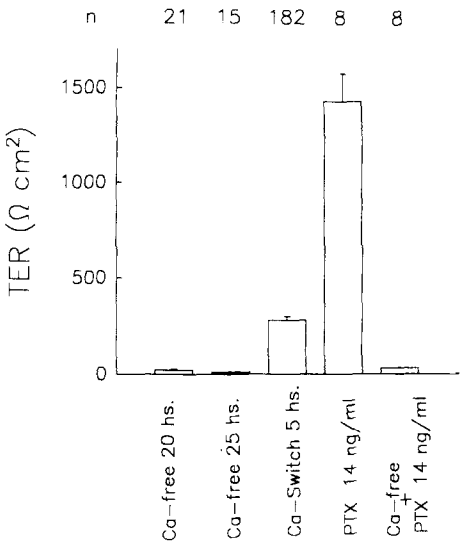

Fig. 1. Effect of PTX on the value of TER at the 5th hr of the Ca switch. MDCK monolayers were plated at confluence on Millipore filters in CDMEM and transferred to Ca-free medium 1 hr later. At the 20th hour of incubation in Ca-free medium monolayers have a negligible TER (first column). Renewal of Ca-free medium for another 5 hr does not modify TER (second column), but switching to a medium with 1.8 mM Ca^{2+} triggers the assembly and sealing of TJs, that confer a TER of 280 ± 20 $\Omega \cdot cm^2$ in 5 hr (third column). PTX added 30 min before and during the Ca switch markedly enhances TER. PTX cannot increase TER by itself, as the addition of 14 ng/ml without Ca^{2+}, fails to seal the junctions (last column)

analysis reveals that, at the concentration used in Fig. 1 (14 ng/ml), pretreatment with PTX produces a 73% inhibition of ADP-ribosylation of the 41 kDa substrate(s) produced by the second PTX exposure.

On the basis of results in Fig. 1, one would expect that stimulators of G-proteins would have an opposite action, i.e., an inhibitory influence on the development of TER. Accordingly, we tested AlF^3 (2 mM) which activates several G-proteins by mimicking the Γ-phosphate group of GTP (Bigay et al., 1985; Blackmore et al., 1985), and carbachol (5 μM), which through muscarinic receptors activates some G-proteins (Nathanson, 1987). Both substances decrease the development of TER ($P \leq 0.001$; Fig. 3). Finally, we used GTP-Γ-s, a non hydrolizable analog of GTP, to test the effect of a continuous activation of G protein(s) on the appearance of the ZO-1 peptide at the cell borders during a Ca-switch. Since GTPΓs is nonpermeable, we treated the cells with digitonin (5 μM, 20 min). Permeabilized monolayers switched from a LC condition to Ca^{2+} containing buffer display clear ZO-1 fluorescence at the cell borders (Fig. 4*B*), while monolayers transferred to Ca^{2+} containing buffer in the presence of GTPΓs do not (Fig. 4*C*). The latter resemble those left in LC

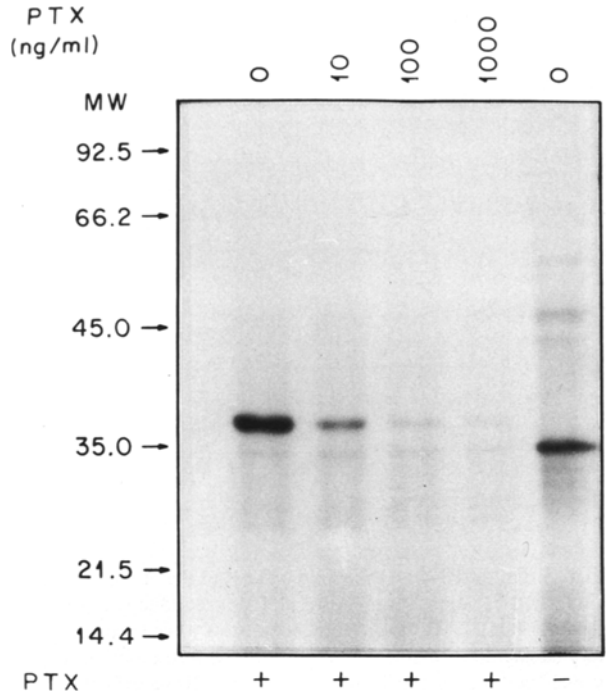

Fig. 2. ADP-ribosylation of a 41-kD protein in MDCK membranes by PTX. Membranes were prepared from MDCK cells that were incubated in the absence (control) or presence of PTX (10, 100, 1000 ng/ml) for 5 hr (*above*). Membranes were then incubated with [α-^{32}P]NAD in the presence or absence (control) of PTX (*below*) as described in Materials and Methods. Markers of molecular weight are given in kilodaltons (arrows)

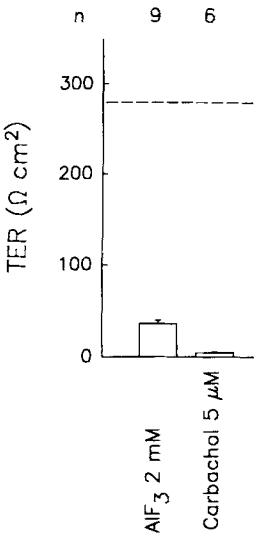

Fig. 3. Effect of activators of G-proteins on the value of TER at the 5th hr of a Ca switch. In this and following figures, the horizontal dashed line represents the control value of TER (3rd column in Fig. 1). AlF_3 (2 mM) and carbachol (5 μM) decrease the value of TER ($P \leq 0.001$)

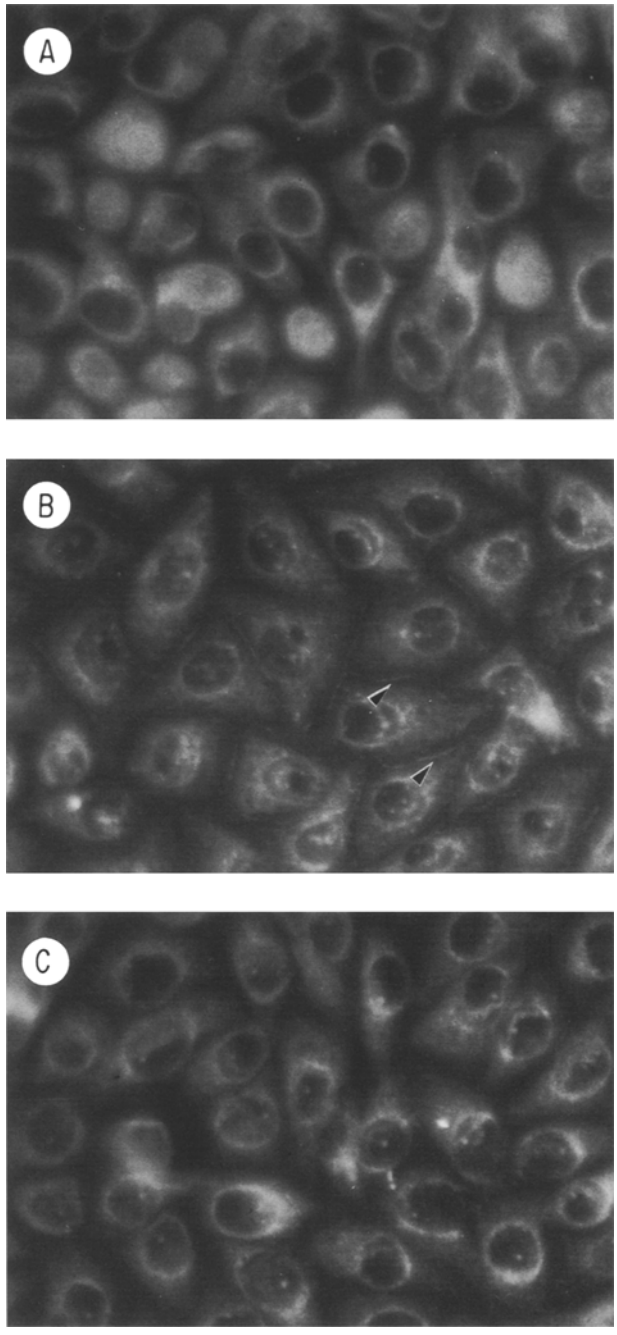

Fig. 4. Effect of GTPΓs on the appearance of the ZO-1 peptide at the plasma membrane during a Ca switch. Monolayers were incubated for 20 hr in LC medium (*A*), then they were permeabilized with 5 μM digitonin in an intracellular buffer (IB, in mM: 2 $MgSO_4$, 2 ATP, 20 PIPES, 0.4 EGTA, 10 glucose, 100 glutamic acid, 100KOH, 29 KCl and 16 NaCl). 20 min later monolayers were transferred for 3.5 hr to IB containing 15 μM Ca^{2+} in the absence (*B*) or presence (*C*) of GTPΓs. Monolayers were fixed in methanol at −20°C for 20 sec, treated with a Mab against ZO-1 and the immunofluorescent pattern was detected with a rhodaminated antibody (goat anti-rat). Arrowheads in *B* show ZO-1 strands. Note the absence of ZO-1 pattern in *C*

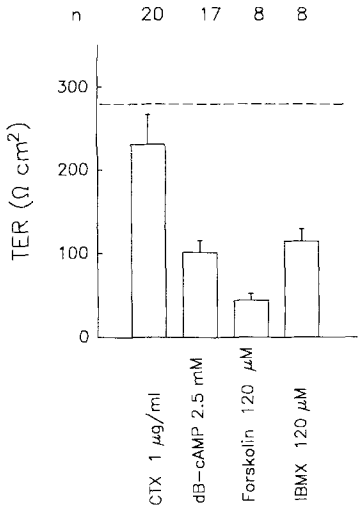

Fig. 5. Effect of adenylate cyclase-related substances on the value of TER at the 5th hour of the Ca switch. Cholera toxin (CTX, 1 μg/ml) produces a nonsignificant decrease of TER (first column). However, a permeable analogue of cAMP (dB-cAMP, 2.5 mM, second column), a stimulator of adenylate cyclase (forskolin, 120 μM, third column), and an inhibitor of phosphodiesterase (IBMX, 120 μM, last column) elicit a profound inhibition ($P \leq 0.001$) of the level of TER

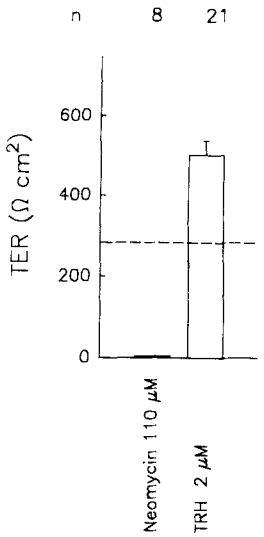

Fig. 6. Effect of phospholipase C-related substances on the value of TER at the 5th hour of the Ca switch. Neomycin (110 μM), a substance that binds to PIP_2 and prevents its conversion to IP_3 plus DAG by phospholipase C, produces a total inhibition ($P \leq 0.001$) of the development of TER. On the contrary, TRH (2 μM), an activator of phospholipase C, elicits a significant stimulation ($P \leq 0.001$)

buffer (Fig. 4*A*). Thus it seems that the stimulation of G-proteins blocks the appearance of ZO-1 at the cell borders as well as the development of TER.

We next attempted to individualize the type of G-protein involved during the Ca switch, starting with the one that activates adenylate cyclase. Figure 5 shows that CTX, which ADP-ribosylates G_s and locks it in its active state, has a small inhibitory effect on TER development. On the other hand, dB-cAMP (a permeable analogue of cAMP), forskolin (an activator of adenylate cyclase), and IBMX (a phosphodiesterase inhibitor) produce significant decreases ($P \leq 0.001$) of TER.

Another G-protein which could participate in the enhancement of TER shown in Fig. 1 is the one modulating PLC. This enzyme, which converts PIP_2 into IP_3 plus DAG (Berridge & Irvine, 1989), may participate in the assembly and sealing of TJs, because when it is inhibited by neomycin (Silvka & Insel, 1988) TER remains low (Fig. 6). Conversely, when it is stimulated by TRH (Martin et al., 1986) the value of TER increases significantly ($P \leq 0.001$).

The chain of reactions triggered by phospholipase C includes a stimulation of PKC by the DAG resulting from PIP_2 hydrolysis. Figure 7 shows that diC8, an activator of PKC added 30 min before the Ca switch and present throughout, elicits a modest enhancement of TER (1st column). However, the effect of this substance may be brief and reversible due to its rapid metabolism to phosphatidic acid (May et al., 1986). Therefore, we also performed experiments adding fresh drug every hour (2nd column) or protecting it with EGD (3rd column). Under these circumstances diC8 increases TER up to 200% ($P \leq 0.001$). On the other hand, inhibitors of PKC such as polymyxin B and H7, that inhibit an exocytic fusion in chromaffin cells (Knight et al., 1988) produce a clear blockade of the development of TER ($P \leq 0.001$; Fig. 8).

The dependence of assembly and sealing on the availability of Ca^{2+} suggests that CaM may also be involved. Figure 9 shows that TFPZ and CMZ, which are potent inhibitors of this protein (Wrenn et al., 1981; Mazzei et al., 1984) impair the development of TER. CaM may in principle participate in the exocytic fusion that incorporates junctional component to the surface membrane, in a way similar to its participation in adrenalin secretion by chromaffin cells (Knight et al., 1988). The inhibition of CaM by CMZ (Fig. 10) blocks the development of the ring of microfilaments, that remain in the nuclear surroundings, as if Ca^{2+} were not added.

We wondered whether the mechanisms controlling the assembly and sealing of TJs may also participate in the control of its tightness in mature monolayers. Accordingly, we also tested the different substances shown in the experiments above presented on mature monolayers of MDCK cells, i.e., monolayers that were plated at confluence and exposed to 1.8 mM Ca^{2+} continuously (Fig. 11). The

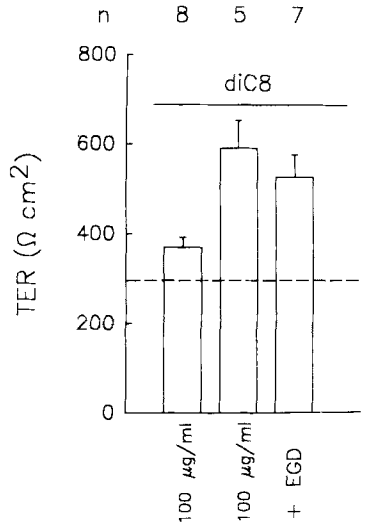

Fig. 7. Effect of a stimulator of PKC on the value of TER at the 5th hour of the Ca switch. 100 μg/ml of diC8, an analog of DAG enhances TER (first column), particularly when it is added fresh every hour (second column) or its metabolization is prevented with EGD (third column)

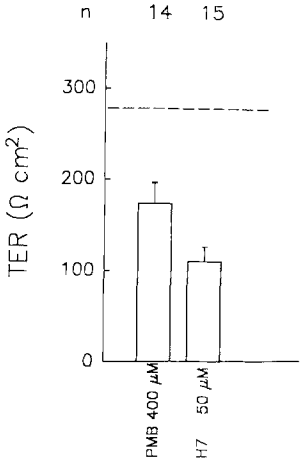

Fig. 8. Effect of inhibitors of PKC on the value of TER at the 5th hour of the Ca switch. PMB (first column) and H7 (second column) produce an inhibition of the development of TER

control value of TER in these monolayers (480 ± 22 [123] Ω · cm²) is significantly ($P < 0.001$) higher than the one achieved at the 5th hour of the Ca switch (280 ± 20 [182] Ω · cm²). Interestingly, 140 ng/ml of PTX, 2 μM TRH and 2 mM AlF_3 produce a significant effect ($P \leq 0.001$) on the value of TER, suggesting TJs in already established epithelia remain sensitive to modulation by G-proteins. On the contrary, stimulators of AC, inhibitors of PKC and CaM exhibit no effect (*data not shown*).

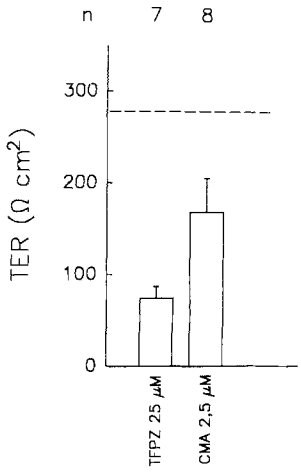

Fig. 9. Effect of CaM inhibitors on the value of TER at the 5th hour of the Ca-switch. TFPZ (first column) and CMZ (second column) inhibit the development of TER

Discussion

We suspected the existence of a delicate mechanism that controls the TJ, because: (i) the effect of Ca^{2+} on junctional assembly and sealing, that depends on intracellular events (e.g. distribution of the cytoskeleton, exocytic fusion) is exerted mainly from the extracellular side (González-Mariscal et al., 1990); (ii) TJs do not seal if cell-attaching molecules are blocked with specific antibodies (Behrens et al., 1985; Gumbiner & Simmons, 1986); (iii) attachment of these molecules in the presence of Ca^{2+} depends on the integrity of their cytoplasmic portion (Ozawa, Baribault & Kemler, 1989); (iv) the degree of tightness varies from one epithelium to another over several orders of magnitude (Cereijido et al., 1988, 1989); and (v) the degree of sealing in a given epithelium may vary in response to a variety of physiological, pharmacological and even pathological conditions (Tice, Wolman & Carter, 1975; Karnaky et al., 1976; Bentzel et al., 1980; Martinez-Palomo et al., 1980; Madara, 1983; Chevalier, Bourguet & Pinto da Silva, 1985; Madara & Pappenheimer, 1987; Lowe et al., 1988). This is now supported by the observation made in this work that the stimulation or inhibition of G-proteins, PLC, PKC, CaM and AC produce a profound effect on the assembly and sealing of the TJ.

Our present data are an initial attempt to characterize the processes involved in the assembly and sealing of TJ at the level of signal transduction. The data with forskolin, CTX, and db-cAMP suggest that activation of adenylate cyclase and the subsequent accumulation of cyclic AMP markedly diminish the development of TER putatively via activation of protein kinase A.

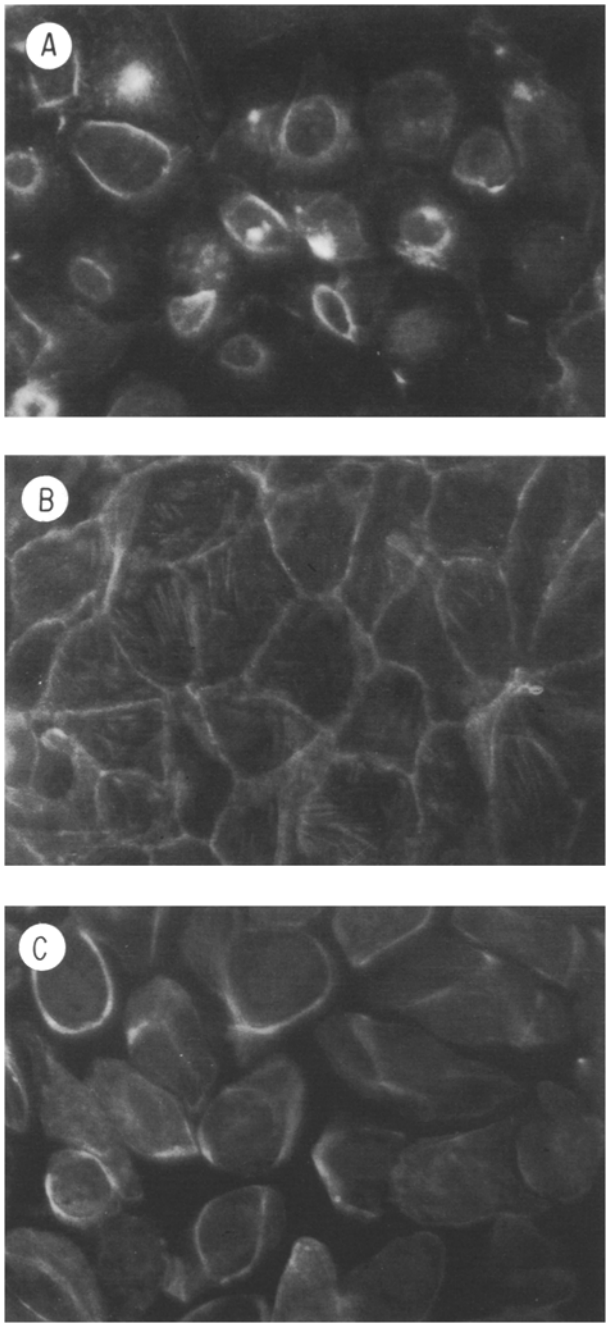

Fig. 10. Effect of CaM inhibition on the pattern of actin filaments in monolayers of MDCK cells. MDCK cells were incubated for 20 hr in LC medium (*A*), transferred for 5 hr to NC medium in the absence (*B*) or presence of 2.5 μM CMZ (*C*). Indirect fluorescence staining of actin filament with phaloidinerhodamine was performed as described in Materials and Methods

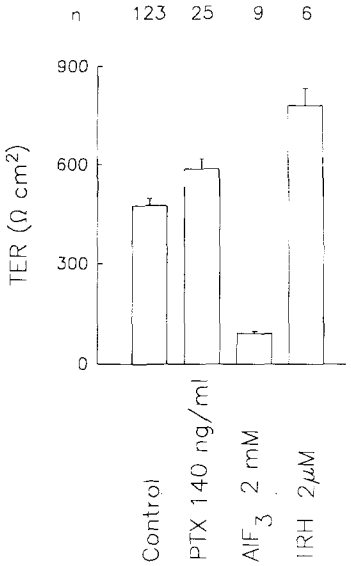

Fig. 11. Effect of different substances on the value of TER of mature monolayers. MDCK cells were plated at confluence and incubated for 20 hr in CDMEM with 1.8 mM Ca^{2+}. Drugs were added at this time and remained present throughout. TER was recorded 5 hr later. PTX and TRH increase and AlF_3 decreases the TER

On the contrary, the use of synthetic diacylglycerol, inhibitors of PKC and agonists, such as TRH that stimulate PKC via receptor-mediated activation of phosphoinositide turnover, are consistent with the view that activation of PKC enhances the development of TER. Thus, it seems that PKC and protein kinase A reciprocally regulate TER development.

It is well known that petussis toxin ADP-ribosylates G_i and blocks receptor-mediated inhibition of adenylate cyclase (García-Sáinz, 1985; Enjalbert et al., 1986; Linden & Delahunty, 1989; Bizzari et al., 1990). It seems unlikely that the increase in TER development induced by the toxin could be explained by this action. However, other G-proteins are also substrates of PTX. It has been suggested that some receptors inhibit PLC via a PTX-sensitive G-protein, termed G_{pi} (Linden & Delahunty, 1989; Bizzarri et al., 1990). It is possible therefore, that PTX may exert its effect on TER development by blocking a constraint on PLC exerted by G_{pi}.

Although clearly speculative, we would like to suggest the following sequence of events in the functional assembly and sealing of TJs during a Ca switch (Fig. 12): (i) Ca^{2+} activates uvomorulin (UV) and other Ca-dependent cell adhesion molecules, which are not located at the TJ itself, but at the *zonula adherens* (Boller, Vestweber & Kemler, 1985; Gumbiner, Stevenson & Grimaldi, 1988). G-proteins may in principle transduce signals from cell-cell contacts to PLC. (ii) PLC produces DAG and IP_3, which in turn releases Ca^{2+} from intracellular reservoirs (Pidikiti et al., 1985). Intracellular Ca^{2+} and DAG

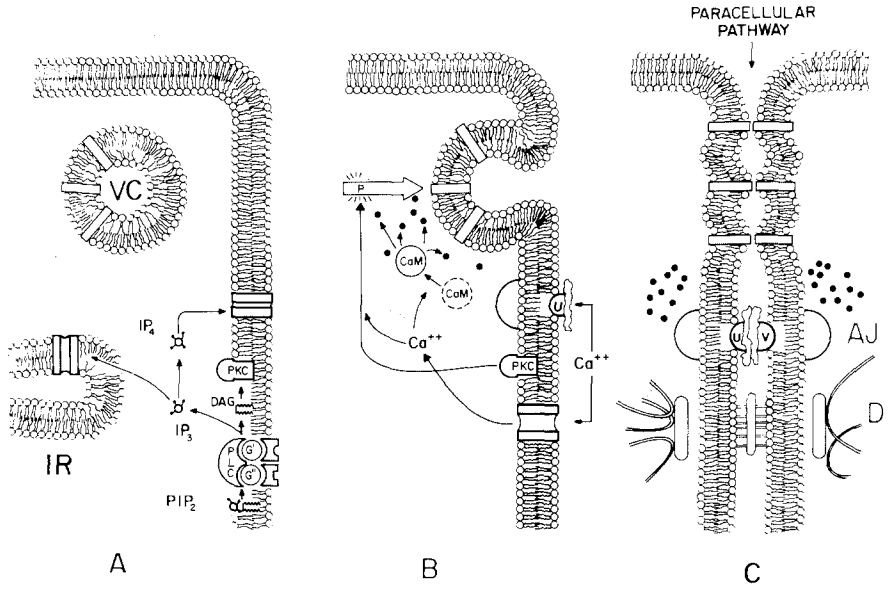

Fig. 12. Highly schematic view of an epithelial cell in the region where TJs develop. (*A*) Cells incubated for 20 hr in Ca^{2+} free media have a cytoplasmic vesicular compartment (*VC*) where junctional components might be stored. PLC is connected to membrane receptors via two G-proteins (*G'* and *G''*). Upon activation it converts PIP_2 into IP_3 and DAG. IP_3 is able to mobilize Ca^{2+} from internal reservoirs (*IR*) and DAG activates PKC. However, the concentration of Ca^{2+} in the bathing solution (1–4 μM) and in the cytoplasm (20 nM) is too low to trigger cell-cell contacts, activate PLC and induce junction formation. (*B*) Upon switching the monolayers to Ca-containing medium,this ion acting on the extracellular side activates a cascade of reactions through G-proteins, PLC and PKC that might induce the exocytic fusion and insertion of junctional components. Activation of CaM by Ca^{2+} would induce arrangement of actin microfilaments into a continuous ring that circles the cell (represented as dots in transversal section). (*C*) Once a ring of actin filaments has formed, and cell-cell contacts at the adherens junction (*AJ*) are established, the TJ develops and the monolayer acquires a TER

activate PKC (Lapetina, Watson & Cuatrecasas, 1984). (iii) Ca^{2+} may also trigger the insertion of ZO-1, monomeric protein peripherally associated with the TJ and phosphorylated at serine residues (Anderson et al., 1988; Stevenson et al., 1989). (iv) The insertion of ZO-1 may be elicited through an exocytic fusion, as González-Mariscal et al. (1990) have shown that during the Ca switch the surface membrane increases by 22%. (v) Ca^{2+} may also activate CaM, as we observe that the inhibition of this protein by CMZ and TFPZ prevents junctional sealing (Fig. 11), and CMZ impairs also the organization of the actin filament ring near the cell-cell contact (Fig. 10).

Kin, Kin and Rhee (1989) have shown that stimulation of AC in blood platelets inhibits PLC. A similar effect on the PLC of MDCK cells may constitute a feedback mechanism, which would enable AC to participate as a negative modulator in the physiological regulation of the assembly and sealing of TJs.

This cellular control of the assembly and sealing of the TJ is in keeping with recent evidence on the regulation of other types of intercellular contacts, such as the involvement of PKC in the translocation of desmoplakins during the formation of desmosomes (Sheu, Kitajima & Yaoita, 1989) and the insertion of E-cadherin during the triggering of compaction of mouse embryo (Winkel et al., 1990). It is also known that the interaction of the cell with collagen stimulates the activity of PKC (Watson et al., 1985; Fuse, Iwanaga & Tai, 1989) and that this interaction affects also the distribution of junction-associated proteins, such as ZO-1 (Wang, Ojakian & Nelson, 1990).

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