Calcium Site Specificity
Early Ca^{2+}-related Tight Junction Events

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ABSTRACT The molecular mechanisms by which Ca^{2+} and metal ions interact with the binding sites that modulate the tight junctions (TJs) have not been fully described. Metal ions were used as probes of these sites in the frog urinary bladder. Basolateral Ca^{2+} withdrawal induces the opening of the TJs, a process that is abruptly terminated when Ca^{2+} is readmitted, and is followed by a complete recovery of the TJ seal. Mg^{2+} and Ba^{2+} were incapable of keeping the TJ sealed or of inducing TJ recovery. In addition, Mg^{2+} causes a reversible concentration-dependent inhibition of the Ca^{2+}-induced TJ recovery. The effects of extracellular Ca^{2+} manipulation on the TJs apparently is not mediated by changes of cytosolic Ca^{2+} concentration. The transition elements, Mn^{2+} and Cd^{2+}, act as Ca^{2+} agonists. In the absence of Ca^{2+}, they prevent TJ opening and almost immediately halt the process of TJ opening caused by Ca^{2+} withdrawal. In addition, Mn^{2+} promotes an almost complete recovery of the TJ seal. Cd^{2+}, in spite of stabilizing the TJs in the closed state and halting TJ opening, does not promote TJ recovery, an effect that apparently results from a superimposed toxic effect that is markedly attenuated by the presence of Ca^{2+}. The interruption of TJ opening caused by Ca^{2+}, Cd^{2+}, or Mn^{2+}, and the stability they confer to the closed TJs, might result from the interaction of these ions with E-cadherin. Addition of La^{3+} (2 μM) to the basolateral Ca^{2+}-containing solution causes an increase of TJ permeability that fully reverses when La^{3+} is removed. This effect of La^{3+}, observed in the presence of Ca^{2+} (1 mM), indicates a high La^{3+} affinity for the Ca^{2+}-binding sites. This ability of La^{3+} to open TJs in the presence of Ca^{2+} is a relevant aspect that must be considered when using La^{3+} in the evaluation of TJ permeability of epithelial and endothelial membranes, particularly when used during in vivo perfusion or in the absence of fixatives.

KEY WORDS: tight junction • calcium • cadmium • lanthanum • E-cadherin

INTRODUCTION

Ca^{2+} is essential for cells to maintain intercellular contacts. When the extracellular Ca^{2+} is removed, the cell–cell connections generally become loose and multicellular organizations are destroyed. A number of studies emphasize the role of extracellular Ca^{2+} on the stability of mature tight junctions (TJs)[1] in natural epithelia (Sedar and Forte, 1964; Hays et al., 1965; Galli et al., 1976; Meldolesi et al., 1978; Pitelka et al., 1983; Palant et al., 1983) and on the development of new TJs in cell cultures in confluence (Martinez-Palomo et al., 1980; Cereijido et al., 1980, 1981; González-Mariscal et al., 1985). The removal of extracellular Ca^{2+} causes the opening of previously formed TJs and prevents de novo formation of TJs in confluent cell monolayers. Notwithstanding several studies addressing the role of extracellular Ca^{2+} in the dynamics of the TJs, major questions are still pending. The relative importance of extracellular (Gorodeski et al., 1997; Contreras et al., 1992; González-Mariscal et al., 1990) versus intracellular (Bhat et al., 1993; Jovov et al., 1994; Stuart et al., 1994) Ca^{2+} concentration on the control of TJs is not yet clearly characterized. The cell adhesion molecule E-cadherin (uvomorulin) (Gumbiner et al., 1988), which is particularly rich at the zonula adhaerens (Boller et al., 1985), plays a key role as the extracellular Ca^{2+} binding molecule that modulates the formation and maintenance of the epithelial junctional complex (Gumbiner et al., 1988). Ca^{2+} influences the conformation of E-cadherin and stabilizes it in its adhesive state (Ringwald et al., 1987). In addition, the interaction of Ca^{2+} with E-cadherin is transduced across the cell membrane by a cascade of reactions involving phospholipase C, G proteins, protein kinase C, and calmodulin (Balda et al., 1991, 1993). The structural and electrostatic mechanisms used by the Ca^{2+} binding sites of E-cadherin to provide Ca^{2+} specificity are not yet fully understood, as compared with the knowledge on the EF-hand-like sites (Snyder et al., 1990), in part because insufficient information is available regarding the ion specificity of the

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[1] *Abbreviations used in this paper:* SCC, short-circuit current; TER, transepithelial electrical resistance; TJ, tight junction.

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Ca^{2+} binding sites. Only recently has the structure of the epithelial cadherin domain responsible for selective cell adhesion been identified. The Ca^{2+} binding site of the CAD1 domain of E-cadherin was inferred by nuclear magnetic resonance identification of the amino acid residues whose backbone ^{13}CO, ^{15}N, or ^{1}HN chemical shifts differed between Ca^{2+}-bound and -free forms (Overduin et al., 1995). A negatively charged pocket is formed by three sequences of residues with the side chains of the highly conserved Glu^{11}, Glu^{69}, and Asp^{100} well positioned to ligate Ca^{2+} (Overduin et al., 1995). In addition, the homophilic specificity surface is also sensitive to Ca^{2+} ligation through His^{79} and Met^{92}, indicating that the Ca^{2+}-induced conformational effect on the homophilic specificity surface may reflect a mechanism by which Ca^{2+} levels regulate the adhesiveness of cadherins (Overduin et al., 1995).

Multiple factors (number, type, and geometry of ligands, electrostatic interactions, cavity size and deformability of the site, dehydration of metal and ligand) are among the variables that must be considered when the metal ion selectivity of protein Ca^{2+} sites are analyzed (Snyder et al., 1990). The exclusion of Mg^{2+} from many protein Ca^{2+} sites, for example, can be explained in part by the fact that Mg^{2+} prefers a coordination number of six and uses nitrogen as a ligand more frequently (Einspahar and Bugg, 1984; Martin, 1984), while coordination by seven oxygens is observed in protein Ca^{2+} sites (Snyder et al., 1990; Strynadka and James, 1989).

In a previous study in the frog urinary bladder (Lacaz-Vieira and Kachar, 1996), it was shown that apical Ca^{2+} may activate the TJ sealing mechanism, an effect that is not impaired by the presence of Ca^{2+} channel blockers (nifedipine, verapamil, Mn^{2+}, or Cd^{2+}) in the apical solution, indicating that junction resealing in the frog urinary bladder does not depend on Ca^{2+} entering the cells through the apical membrane. Most likely, this effect results from Ca^{2+} entering partially disrupted TJs, reaching the zonula adhaerens Ca^{2+} receptors (E-cadherins). It was also shown that protein kinase C plays a significant role in the control of TJ assembly in the frog urinary bladder since the PKC inhibitor (H7) and the activator (diC8) markedly affect TJ recovery after they are disrupted by apical hypertonicity.

The present study addresses the interactions of the metal ions with the binding sites that affect the function of the TJs in order to characterize their selectivity.

MATERIALS AND METHODS

Urinary bladders of the frog *Rana catesbeiana* were used. Animals were anesthetized by subcutaneous injection of a 2% solution of 3-aminobenzoic acid ethyl ester (methanesulfonate salt) (Sigma Chemical Co., St. Louis, MO) at a dose of 1 ml/100 g body wt. The abdominal cavity was opened, a cannula was passed through the cloaca, and the urinary bladder was inflated with 15–20 ml of air according to the animal size. Plastic rings of 20-mm diameter were glued to the serosal surface of the bladder with ethylcyanoacrylate adhesive (Super Bonder; Loctite, São Paulo, Brazil). The fragment of tissue framed by the plastic ring was excised and immersed in Ringer solution. Subsequently, it was mounted in a modified Ussing's chamber (Castro et al., 1993), exposing an area of 0.5 cm^2. Hemichambers with a recessed rim filled with high viscosity silicone grease (High Vacuum Grease; Dow-Corning Corp., Indianapolis, IN) prevented tissue edge damage (Lacaz-Vieira, 1986). Each chamber compartment was perfused with a continuous flow of solution (up to 25 ml/min) driven by gravity from reservoirs through plastic tubings. Unstirred layers on the surfaces of the tissue were minimized by directing the incoming fluid towards the tissue surfaces. Each compartment was drained through a spillway open to the atmosphere so that the pressure inside each compartment was kept fairly constant at the atmospheric level. Rapid solution changes were obtained without interruption of voltage clamping by switching the inlet tubings at their connections with the chamber.

Solutions

Unless otherwise stated, the inner bathing solution was NaCl Ringer's solution. The Ringer's compositions were (mM): NaCl Ringer: 115 NaCl, 2.5 $KHCO_3$, and 1.0 $CaCl_2$. Na_2SO_4 Ringer: 57.5 Na_2SO_4, 2.5 $KHCO_3$, 1.0 $CaSO_4$. NaCl HEPES Ringer: 115 NaCl, 2.5 KCl, 2.0 HEPES. All Ringer's solutions had their pH adjusted to 8.2 after aeration. The apical bathing fluids were simple salt solutions, nonbuffered, prepared with glass-distilled water, having pH ~6.0 and free Ca^{2+} concentration in the range of 1.5 × 1–7 and 2.0 × 1–7 M (Castro et al., 1993). In the beginning of the experiments, the apical solution was 75 mM KCl.

Electrical Measurements

A conventional analog voltage clamp (DVC 1000; WPI, New Haven, CT) was used. Saturated calomel half-cells with 3 M KCl-agar bridges were used to measure the electrical potential difference across the bladder. Current was passed through Ag-AgCl 3 M KCl electrodes and 3 M KCl-agar bridges, adequately placed to deliver a uniform current density across the bladder. The clamping current was continuously recorded by a strip-chart recorder. Clamping current and voltage were also digitized through an analog-to-digital converter (Digidata 1200 and Axotape 2.0; Axon Instruments Inc., Foster City, CA) and stored in a computer for further processing. A digital Gaussian Filter (Colquhoun and Sigworth, 1983) was used to remove high frequency noise of the baseline of all records used in the figures. This digital filter forms output values γ_i from input values χ_i by performing the arithmetic mean of three consecutive current values, so that

$$\gamma_i = \left(\sum_{i-1}^{i+1} \chi_i \right) / 3 .$$

Isotopic Flux Measurements

^{14}C-Sucrose (Amersham International, Little Chalfont, UK) was added to the apical or basolateral solution, and the fluid was recirculated with a peristaltic pump at a rate of 5 ml/min. An equilibration period of at least 20 min, during which the solution in the opposite compartment was continuously renewed, was allowed before the sampling in this compartment. During the sampling period, the flow of solution through the compartment was stopped and the solution was stirred by a plastic propeller driven by an electric motor. At the end of the collection interval, the so-

lution was removed for radioactivity assay. Subsequently, the compartment was refilled and a new sampling period started.

TJ Blockade by the Selective Deposition of $BaSO_4$

TJ blockade was induced according to a previously described method (Castro et al., 1993). Tissues were bathed on the basolateral side by Na_2SO_4 Ringer's solution. To induce the blockade by the selective deposition of $BaSO_4$ in the TJs, the apical bathing fluid was replaced by a solution of $BaCl_2$ (50 mM) and a positive clamping potential (+50 mV) was applied across the tissue to force migration of Ba^{2+} and SO_4^{2+} against each other and the formation of $BaSO_4$ precipitate at the TJ level.

Chemicals

All chemicals were obtained from Sigma Chemical Co.

Statistics

The results are presented as mean ± SEM. Comparisons were carried out using Student's paired *t* test. When more than two groups were compared, significance was determined by two-way analysis of variance followed by appropriate posttest comparison. The *P* values cited include Bonferroni's correction (Neter and Wasserman, 1974).

RESULTS

The experiments were carried out in short-circuited frog urinary bladders bathed on the basolateral side by NaCl-Ringer's solution (or a different Ringer's solution according to the protocol), and on the apical side, in most cases, by a simple solution of KCl (75 mM). The absence of Na^+ in the apical solution aimed at abolishing the short-circuit current as well as the role of transcellular Na^+ conductance to the overall tissue electrical conductance, so that changes in the transepithelial electrical resistance (TER) reflected changes in the electrical resistance of the tight junctions, as in other tight epithelia (Jovov et al., 1994; Wills and Millinoff, 1990).

Transepithelial Electrical Resistance

TER is shown in $\Omega\ cm^2$, calculated from the deflections of the clamping current induced by shifts of the clamping potential of 300 ms duration, ±1 mV amplitude at 15-s intervals, as $TER = \Delta V_t/\Delta I_t$, where ΔV_t and ΔI_t are the changes in the electrical potential difference across the tissue and clamping current, respectively. I_t, clamping current in $\mu A/cm^2$. Positive (or inward) current corresponds to the transport of positive charges across the tissue, from the apical to the inner bathing solution. V_t, electrical potential difference across the tissue (millivolts). The potential of the apical solution is referred to that of the inner solution.

The general protocol consisted in analyzing the interactions of metal ions with the binding sites that affect the TJ permeability according to a Ca^{2+}-switch assay that consisted of a two-step process: (*a*) increase of TJ permeability, characterized by a drop in TER, was induced by removing Ca^{2+} from the basolateral solution. (*b*) TJ recovery, characterized by return of TER to initial values, was achieved by the reintroduction of Ca^{2+} into the basolateral solution. Small, short-term perturbations of the TJs were induced to prevent or minimize tardy regulatory responses that might complicate the results. This is exemplified by the fact that the rate of Ca^{2+}-induced TJ recovery depends on the degree of TJ opening, which, in turn, depends on the time the bladders were without Ca^{2+}, in agreement with observations in MDCK (Martinez-Palomo et al., 1980) and A6 (Jovov et al., 1994) cell monolayers. To cope with this problem, the drop of TER in response to Ca^{2+} withdrawal was normally terminated by the readmission of Ca^{2+} to the basolateral fluid when TER reached values close to 250 $\Omega\ cm^2$. The experiments were carried out, unless specified, with nominally Ca^{2+}-free apical solution. The presence of Ca^{2+} in the apical solution is not essential for stability of TJs in A6 cell monolayers (Jovov et al., 1994) or in the frog urinary bladder (Lacaz-Vieira and Kachar, 1996).

Effect of Basolateral Ca^{2+} on TER

Ca^{2+} removal from the basolateral solution (NaCl, Na_2SO_4, or NaCl HEPES Ringer's solution) induces, after a lag time (generally between 30 s and 3 min), a pronounced drop of TER. Once started, the decline of TER shows a rapid progress. Return to Ca^{2+} promptly stops the decline of TER and triggers a full recovery. In the example of Fig. 1 *A*, the onset of TER decline has a lag time of 90 s and TER drops to 2% of the initial value in 160 s. Mean values of TER for a group of eight bladders bathed on the apical side by 75 mM KCl and by NaCl Ringer's solution on the basolateral side are: initial condition, 11,729.5 ± 1,532.5 $\Omega\ cm^2$; 120 s after Ca^{2+} removal from the basolateral medium, 207.3 ± 32.3 $\Omega\ cm^2$; after full recovery of TER in response to the reintroduction of Ca^{2+} into the basolateral medium, 11,567.1 ± 1,667.0 $\Omega\ cm^2$. The changes in short-circuit current (SCC) that take place in conjunction with the changes in TER result from the movement of ions (mostly Na^+ and K^+) along the paracellular pathway driven by their concentration differences in the bathing solutions.

A stepwise reduction of basolateral Ca^{2+} concentration (by addition of EGTA) does not result in a decrease of TER until $[Ca^{2+}]_{bl}$ reaches values in the range of 70–100 μM. A further decrease in concentration resulted in a pronounced decline of TER. The dependence of steady state values of TER on $[Ca^{2+}]_{bl}$ is sigmoidal and conforms with the Hill equation (Rodwell, 1996): $TER_{[Ca]} = TER/(1 + [K_m/[Ca]]^n)$, with a K_m value of 62 ± 28 μM and a Hill coefficient (*n*) of 8.6 ± 0.9, indicating a steep dependence of TER on $[Ca^{2+}]_{bl}$. $TER_{[Ca]}$ is the value of TER at any given serosal Ca^{2+}

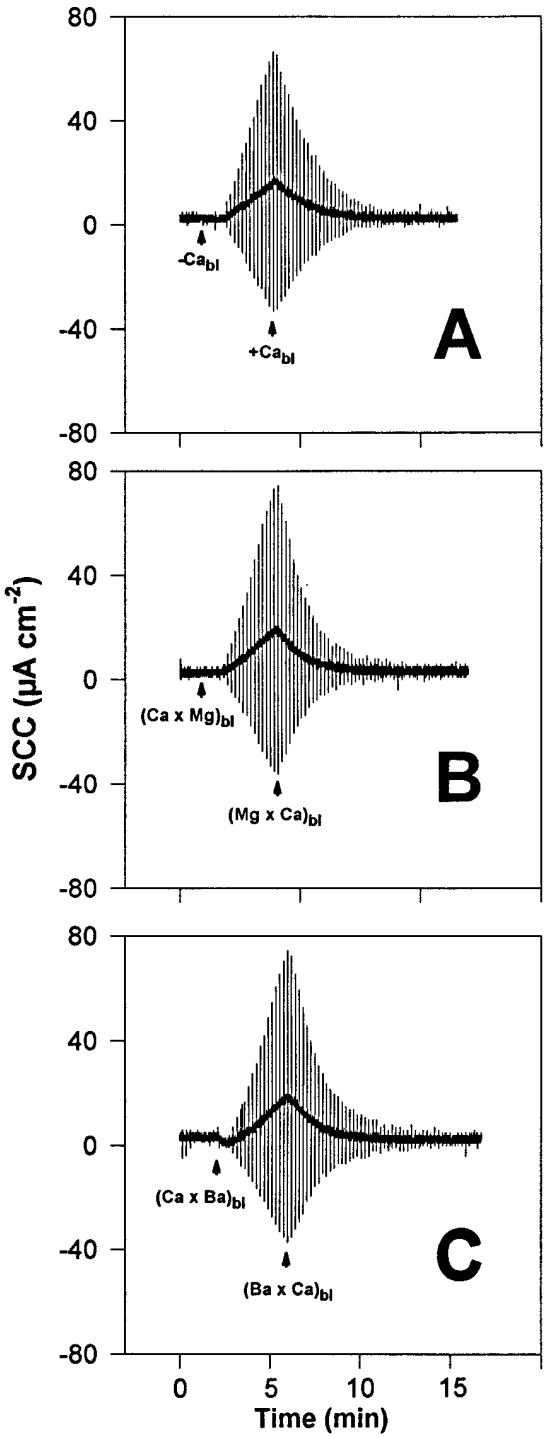

FIGURE 1. Representative experiments (of a group of eight tissues) carried out in the same piece of bladder showing the effect of Ca^{2+} withdrawal from the basolateral solution on TER and the reversibility of the process. The urinary bladder was short-circuited and bathed by 75 mM KCl on the apical side and by NaCl Ringer's solution on the basolateral side. (*A*) The basolateral Ca^{2+} was removed ($-Ca_{bl}$) and reintroduced ($+Ca_{bl}$). (*B*) The basolateral Ca^{2+} was equimolarly replaced by Mg^{2+} ($(Ca \times Mg)_{bl}$); subsequently, this change was reversed ($(Mg \times Ca)_{bl}$). (*C*) The basolateral Ca^{2+} was equimolarly replaced by Ba^{2+} ($(Ca \times Ba)_{bl}$); subsequently, this change was reversed ($(Ba \times Ca)_{bl}$). The vertical deflections of SCC, caused by pulses of ±1 mV in the clamping potential, are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

concentration; TER is the value of TER at 1 mM serosal Ca^{2+} concentration; K_m is the serosal Ca^{2+} concentration that reduces TER to 50% of the value at normal Ca^{2+} concentration.

The drop of TER that follows basal Ca^{2+} withdrawal is caused by a decrease of TJ permeability since it is accompanied by a significant increase of tissue permeability to ^{14}C-sucrose that fully reverses upon reintroduction of Ca^{2+}. The sucrose influx (J^{in}), which reflects the magnitude of the paracellular permeability, increased from (*a*) 0.65 ± 0.06 pmol cm^{-2} min^{-1} in the control condition (75 mM KCl on the apical side and NaCl Ringer's on the basolateral side) to (*b*) 3.74 ± 0.09 pmol cm^{-2} min^{-1}, 5 min after Ca^{2+} removal from the basolateral solution, and returned to a steady value of (*c*) 0.68 ± 0.07 pmol cm^{-2} min^{-1} 10 min after addition of Ca^{2+} to the basolateral solution. Statistical comparison: *a–b*, $P < 0.01$; *a–c*, P = NS ($n = 6$).

To circumvent a conceivable objection that sucrose flux measurements, which involve long periods of time, might not provide a clear indication that the initial drop of TER in response to basolateral Ca^{2+} withdrawal results from an increase of TJ permeability, additional experiments were performed in which open TJs were blocked by the selective deposition of $BaSO_4$ (Castro et al., 1993). The urinary bladders were bathed on the basolateral side by a sulfate-containing solution (Na_2SO_4 Ringer's, see MATERIAL AND METHODS) to cause precipitation of $BaSO_4$ in the open TJs when $BaCl_2$ is added to the apical compartment. As soon as TER decreased in response to Ca^{2+} withdrawal from the basolateral fluid, the addition of Ba^{2+} to the apical solution leads to a prompt and marked increase of TER that results from the blockade of the permeabilized TJs by precipitation of $BaSO_4$ (Fig. 2). In a control group of bladders bathed by NaCl Ringer's, no effect was observed in response to the addition of Ba^{2+} to the apical solution, excluding the possibility that the increase of TER caused by apical Ba^{2+} resulted from the blockade of a transcellular pathway involving K channels (Van Driessche and Zeiske, 1980). The experiments with Ba^{2+} provide strong evidence that the early drop of TER associated with basolateral Ca^{2+} withdrawal results from a relaxation of the TJ seal.

Role of Cytosolic Ca^{2+} Concentration on TER Responses to Changes in Extracellular Ca^{2+} Concentration

To ascertain the contribution of cytosolic Ca^{2+} concentration on TER responses to changes in extracellular Ca^{2+} concentration, two experimental approaches were used.

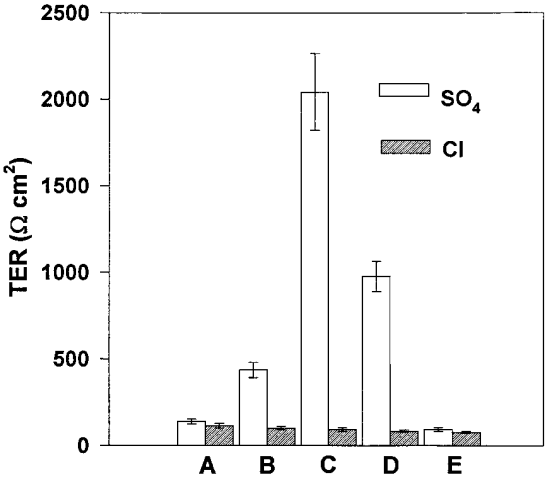

FIGURE 2. TJ blockade by the selective deposition of $BaSO_4$. This maneuver permits us to certify that the early drop of TER that takes place in response to basolateral Ca^{2+} withdrawal results from the opening of TJs. The urinary bladders were initially short-circuited and bathed on the apical side by 75 mM KCl and on the basolateral side by Na_2SO_4 Ringer's (*clear bars*) or NaCl Ringer's (*hatched bars*). (*A*) TER was evaluated 120 s after the beginning of TER drop in response to basolateral Ca^{2+} removal. (*B*) The apical solution was then replaced by 50 mM $BaCl_2$ and TER was evaluated after 20 s. (*C*) Without removing Ba^{2+}, the clamping potential was set to +50 mV and TER was evaluated 60 s later. (*D*) Without removing Ba^{2+}, the clamping potential was returned to 0 mV and, after 20 s, TER was evaluated. (*E*) The apical compartment was rinsed several times with 75 mM KCl and TER was evaluated 10 min later. It can be seen that only tissues bathed on the inner surface by Na_2SO_4 Ringer's solution showed a marked increase of TER in response to the presence of Ba^{2+} in the apical compartment, an increase that is enhanced by the imposition of a +50-mV calming potential that favors the interaction of Ba^{2+} and SO_4^{2+} at the TJ level and the formation of $BaSO_4$ precipitate. $n = 6$.

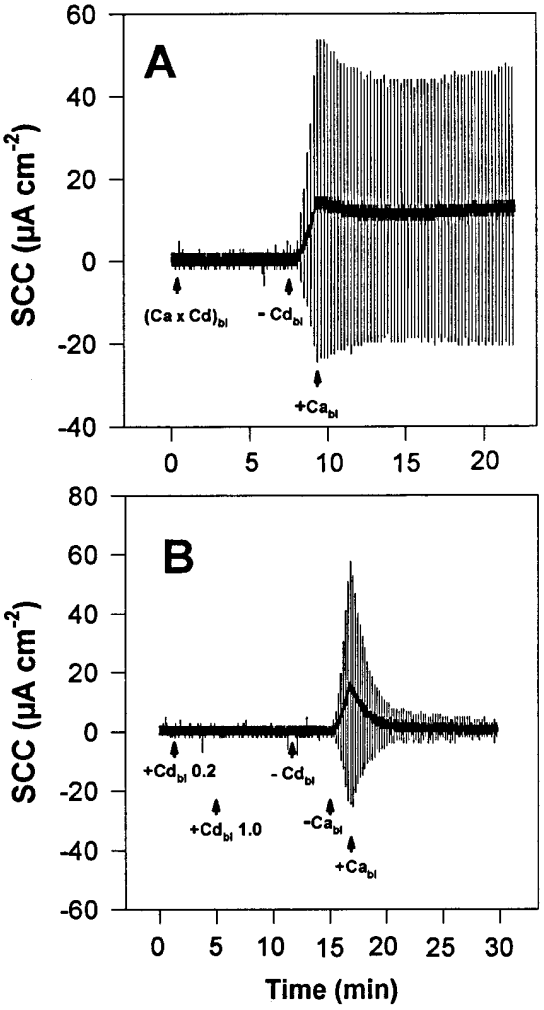

FIGURE 3. Representative experiments (of a group of seven tissues) of the action of Cd^{2+} added to the basolateral solution on the dynamics of TJ opening and closing. The urinary bladders were short-circuited and bathed on the apical side by 75 mM KCl and on the basolateral side by NaCl Ringer's solution. (*A*) Basolateral Ca^{2+} was replaced by 1 mM Cd^{2+} ($(Ca \times Cd)_{bl}$) with no resulting increase of TER. Afterwards, Cd^{2+} was removed from the basolateral solution ($-Cd_{bl}$) leading, after a few seconds, to an increase of TER that can be terminated by reintroduction of Ca^{2+} (1 mM) into the basolateral fluid ($+Ca_{bl}$). No recovery of TER was observed in response to reintroduction of Ca^{2+}, in contrast to control tissues. (*B*) Cd^{2+} was added to the basolateral solution (in the presence of 1 mM Ca^{2+}) at concentrations of 0.2 mM ($+Cd_{bl}$ *0.2*) and 1.0 mM ($+Cd_{bl}$ *1.0*). Subsequently, Cd^{2+} was removed ($-Cd_{bl}$) without any noticeable effect. Afterwards, basolateral Ca^{2+} was also removed ($-Ca_{bl}$), leading to a conspicuous decrease of TER that fully reverts upon readmission of Ca^{2+} into the basolateral fluid ($+Ca_{bl}$). The vertical deflections of SCC, caused by pulses of ±1 mV in the clamping potential, are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

BAPTA-AM. The epithelial cells were loaded with Ca^{2+} chelator by incubating tissues ($n = 5$) with the cell-permeant BAPTA-AM ester (10 μM) on both sides for 20 min. No effect was observed on TER. The chelator was then removed together with Ca^{2+} from the basolateral fluid, leading to a drop of TER that fully recovered upon Ca^{2+} return to the basolateral fluid. The fact that in the presence of an intracellular Ca^{2+} chelator, the introduction of Ca^{2+} into the basolateral fluid triggers TJ recovery is a strong argument in favor of an extracellular effect of Ca^{2+}, most certainly at the level of E-cadherin.

Ionophore A-23187. Two different protocols were tested. In one ($n = 3$), the experiments were performed with an apical solution containing 75 mM KCl, 1 mM Ca^{2+}, and 3 μM A23187. The presence of the ionophore in the apical solution caused only a small decrease of TER that soon stabilized. A subsequent removal of basolateral Ca^{2+} induced a reduction of TER similar to that shown in Fig. 1 *A*. Recovery was obtained by reintroducing Ca^{2+} into the basolateral fluid. In another group of experiments ($n = 5$), the apical solution was 75 mM KCl plus the ionophore (3 μM). TJs were opened by removal of basolateral Ca^{2+}, and then the apical solution was replaced by another containing, in addition to the ionophore, 1 mM Ca^{2+}. Upon addition

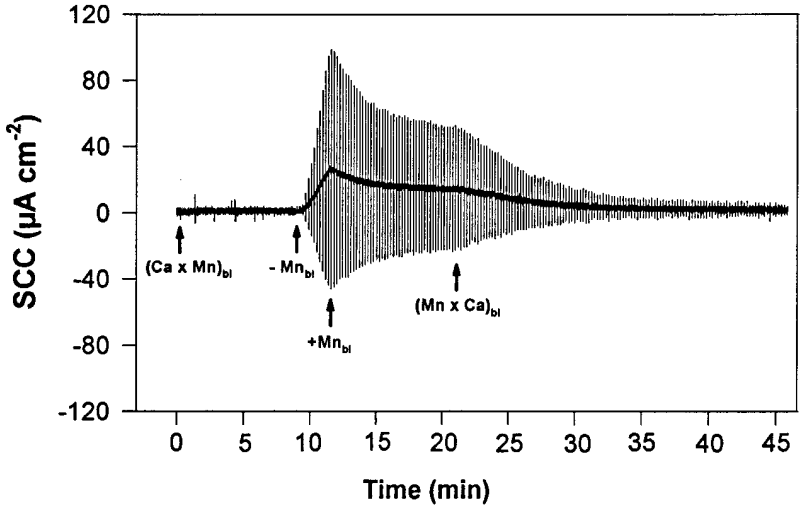

FIGURE 4. Representative experiments (of a group of eight tissues) of the action of Mn^{2+} added to the basolateral solution on the dynamics of TJ opening and closing. The urinary bladders were short-circuited and bathed on the apical side by 75 mM KCl and on the basolateral side by NaCl Ringer's solution. Basolateral Ca^{2+} was replaced by 1 mM Mn^{2+} ($(Ca \times Mn)_{bl}$) with no resulting increase of TER. Afterwards, Mn^{2+} was removed from the basolateral solution ($-Mn_{bl}$) leading, after a few seconds, to an increase of TER. Reintroduction of Mn^{2+} (1 mM) into the basolateral fluid triggers a slow recovery of TER that speeds up considerably when this ion is replaced by Ca^{2+} ($(Mn \times Ca)_{bl}$). The vertical deflections of SCC, caused by pulses of ±1 mV in the clamping potential, are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

of Ca^{2+} to the apical solution, a transient reduction of TER that lasted 1–2 min was observed, followed by a subsequent decline of TER. Return to Ca^{2+} in the basolateral solution then triggered a complete recovery of TER. These experiments suggest that Ca^{2+} entering the cells through the pathways created by the ionophore may transiently trigger TER recovery. However, a complete and stable recovery of the TJ seal was only obtained upon addition of Ca^{2+} to the basolateral solution.

Divalent Metal Ions

These experiments aimed to appraise the degree of interaction of divalent cations with the basolateral Ca^{2+} sites that affect the sealing of the TJs. Two different aspects were analyzed: (*a*) the ability of the metal ion to prevent the opening of the TJs when the metal ion replaced the basolateral Ca^{2+}, and (*b*) the ability of the metal ion to induce the resealing of TJs previously opened by the removal of basolateral Ca^{2+}.

Alkaline earth metals as controls: Mg^{2+} and Ba^{2+}. The equimolar substitution of basolateral Ca^{2+} by Mg^{2+} causes a drop in TER similar to that induced by the removal of Ca^{2+} from the basolateral fluid. Return to Ca^{2+} leads to a full recovery of TER (Fig. 1 *B*). A similar behavior is observed in response to the substitution of the basolateral Ca^{2+} by Ba^{2} (Fig. 1 *C*). These results indicate that Mg^{2+} and Ba^{2+} are ineffective in maintaining the TJ seal in the frog urinary bladder. Experiments (not shown) also indicate that TJs previously opened by Ca^{2+} withdrawal do not close in response to addition of Mg^{2+} or Ba^{2+} (1 mM) to the basolateral medium. Higher concentrations up to 10 mM were tested without effect.

Transition elements: Cd^{2+} and Mn^{2+}. The equimolar substitution of basolateral Ca^{2+} by Cd^{2+} (Fig. 3 *A*) or Mn^{2+} (Fig. 4) does not lead, as observed for the alkaline earth metals, to a reduction of TER, indicating that these transition elements show a Ca^{2+} agonistic effect in short term experiments, characterized by their ability to keep the TJs closed in the absence of basolateral Ca^{2+}. A subsequent withdrawal of Cd^{2+} (Fig. 3 *A*) or of Mn^{2+} (Fig. 4) triggers junction opening, indicated by a decline of TER that follows a time course comparable with that observed in response to basolateral Ca^{2+} removal. TJ opening that follows Cd^{2+} removal is promptly halted upon reintroduction of Ca^{2+} (Fig. 3 *A*) or even Cd^{2+} into the basolateral solution. Recovery is, however, incomplete even in response to Ca^{2+}, suggesting a residual, apparently toxic effect of Cd^{2+}. This toxic effect of Cd^{2+} is greatly reduced or even eliminated if Ca^{2+} is also present, as shown in Fig. 3 *B*. Tissues exposed for several minutes to basolateral Cd^{2+} (1 mM) in the presence of a normal basolateral Ca^{2+} concentration behave, after Cd^{2+} removal, as control tissues not exposed to Cd^{2+}.

The effect of basolateral Mn^{2+} (Fig. 4) is different from that of Cd^{2+} since, in addition to promptly halting the decline of TER, Mn^{2+} leads to a slower but well characterized recovery of TER, a process that is accelerated and reaches completion if Mn^{2+} is replaced by Ca^{2+}.

Apical Ca^{2+} May Reach the Binding Sites that Affect the TJs

The results presented so far show reversible changes of TER due to manipulation of basolateral Ca^{2+} in the absence of apical Ca^{2+}. Similar results can be obtained in the presence of 1 mM Ca^{2+} in the apical solution. Higher concentrations of apical Ca^{2+}, however, may curb the increase of TJ permeability that results from basolateral Ca^{2+} withdrawal. Thus, the presence of 10 mM Ca^{2+} in the apical solution markedly depresses (Fig. 5 *A*) or even abolishes (Fig. 5 *B*) TER decrease in response to basolateral Ca^{2+} withdrawal. A subsequent removal of apical Ca^{2+} speeds up (Fig. 5 *A*) or triggers (Fig. 5 *B*) a TER decrease that had been blocked by the

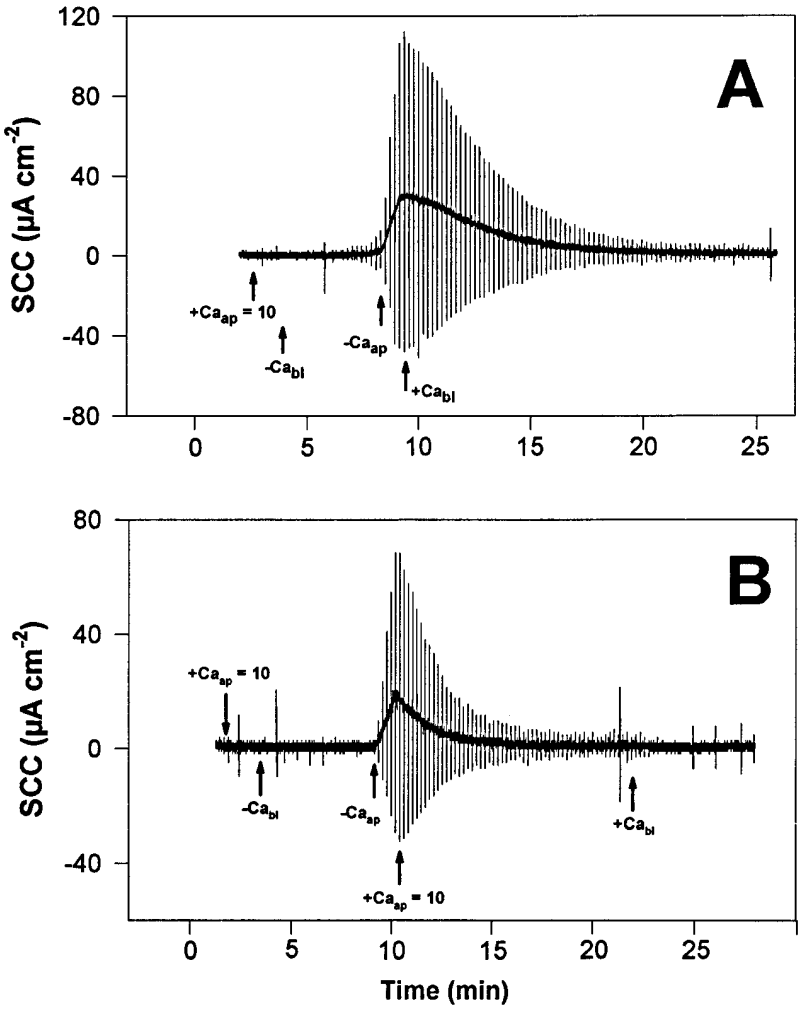

FIGURE 5. Representative experiment (of a group of 10 tissues) of the action of apical Ca^{2+} on the TJ response to withdrawal of basolateral Ca^{2+}. The urinary bladders were short-circuited and bathed on the apical side by 75 mM KCl and on the basolateral side by NaCl Ringer's solution. (*A*) The presence of 10 mM Ca^{2+} in the apical bathing fluid ($+Ca_{ap} = 10$) markedly reduces the rate of TER decrease in response to Ca^{2+} removal from the basolateral solution ($-Ca_{bl}$). Subsequent removal of apical Ca^{2+} ($-Ca_{ap}$) substantially increases the rate of TER decrease. TER fully recovers upon addition of 1 mM Ca^{2+} to the basolateral solution. (*B*) In this bladder, the presence of 10 mM Ca^{2+} in the apical solution ($+Ca_{ap} = 10$) completely abolished the decrease of TER that normally follows the removal of Ca^{2+} from the basolateral solution ($-Ca_{bl}$). The subsequent withdrawal of apical Ca^{2+} ($-Ca_{ap}$) then triggers a drop of TER that starts with an extremely short delay after the Ca^{2+} removal. In the absence of basolateral Ca^{2+}, the introduction of 10 mM Ca^{2+} into the apical solution ($+Ca_{ap} = 10$) leads to an almost complete recovery of TER. A full recovery of TER is attained when 1 mM Ca^{2+} is added to the basolateral fluid. The vertical deflections of SCC, caused by pulses of ±1 mV in the clamping potential, are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

high apical Ca^{2+} concentration. TJ permeability increase induced by withdrawal of basolateral Ca^{2+} stops promptly and reverts almost completely in response to addition of 10 mM Ca^{2+} to the apical solution (Fig. 5 *B*). Ca^{2+} channel blockers (Nifedipine, 1 and 3 μM; Verapamil, 0.3 mM) added to the apical solution have no influence on the effect of a high apical Ca^{2+} concentration, confirming previous findings (Lacaz-Vieira and Kachar, 1996) that the effect of apical Ca^{2+} is not mediated by Ca^{2+} entering the cells through apical Ca^{2+} channels. In conclusion, these results support the notion that apical Ca^{2+}, crossing the open TJs, may reach the binding sites affecting TJ permeability. When a sufficient Ca^{2+} concentration is present in the apical solution, diffusion through normally closed TJs may be sufficient to raise the Ca^{2+} concentration at the binding sites as to overcome the withdrawal of Ca^{2+} from the basolateral solution.

Mg^{2+} Competes with Ca^{2+} for the Binding Sites

These experiments use the fact just described that open TJs allow access of apical ions to the binding sites that affect the TJs. The presence of Mg^{2+} in the apical solution causes a concentration-dependent inhibition of TER recovery in response to Ca^{2+}. The effect of apical Mg^{2+} starts to be noticed at apical concentrations at or above 5 mM. Fig. 6 shows an example in which 20 mM Mg^{2+} in the apical bathing medium practically abolishes the recovery of TER that occurs in response to the reintroduction of basolateral Ca^{2+}. The subsequent withdrawal of Mg^{2+} from the apical solution triggers the TJ recovery process. These results show that Mg^{2+} reversibly competes with Ca^{2+} for the binding sites that control the TJ. It is interesting to observe that a high apical Mg^{2+} concentration inhibits the Ca^{2+}-induced recovery of TER, but not the ability Ca^{2+} has to halt the decrease of TER (Fig. 6).

La^{3+} Causes TJ Opening in the Presence of Ca^{2+}

These experiments were carried out in NaCl HEPES Ringer's solution to prevent precipitation of La^{3+} in bicarbonate Ringer's solution due to the formation of poorly soluble lanthanum bicarbonate. Addition of La^{3+} (2 μM, as $La(NO_3)_3$) to the basolateral Ringer's

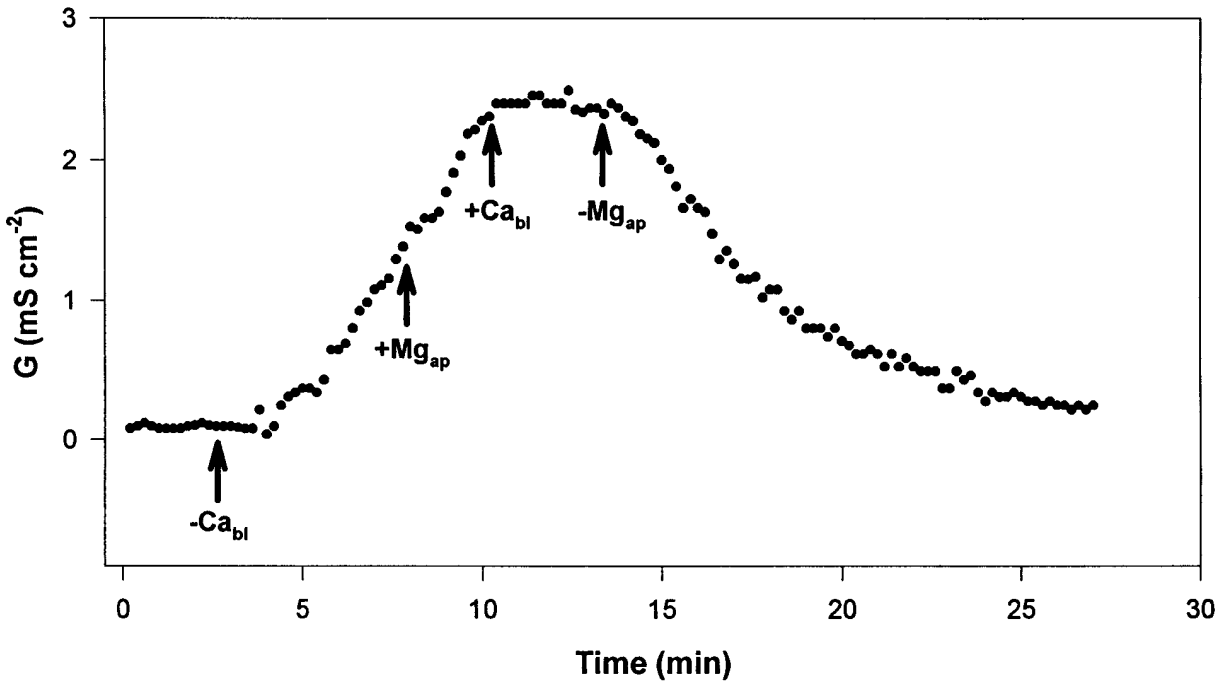

FIGURE 6. Representative experiment (of a group of six) of the effect of apical Mg^{2+} on tissue electrical conductance (G) response to basolateral Ca^{2+} removal ($-Ca_{bl}$) and reintroduction ($+Ca_{bl}$). The urinary bladders were short-circuited and bathed on the apical side by 75 mM KCl and on the basolateral side by NaCl Ringer's solution. During the phase of G increase, the apical solution was replaced by a solution of 55 mM KCl plus 20 mM $MgCl_2$ ($+Mg_{ap}$). Removal of apical Mg^{2+} was obtained by returning to a solution of 75 mM KCl ($-Mg_{ap}$).

solution (in the presence of Ca^{2+}) causes a drop of TER (Fig. 7 *A*) with lag phases and time courses similar to those observed in response to basolateral Ca^{2+} withdrawal (Fig. 7 *B*). A subsequent removal of La^{3+} from the basolateral solution promotes a recovery of TER similar to what is observed when Ca^{2+} is readmitted to the basolateral solution after being previously removed. Higher concentrations of La^{3+} (>1 mM) may slow the quick recovery that follows its removal. This effect of basolateral La^{3+} on TER, observed in the presence of a normal Ca^{2+} concentration, characterizes a Ca^{2+} antagonistic effect of La^{3+}.

Addition of La^{3+} (2 μM as $La(NO_3)_3$) to the apical solution (75 mM KCl) causes no effect on TER as it does when added to the basolateral solution. This indicates that the normally closed TJs of the urinary bladder are sufficiently restrictive to hamper the movement of the trivalent La^{3+} ion, preventing it from reaching the Ca^{2+} binding sites that, as previously seen, are readily accessible to La^{3+} from the basolateral aspect of the tissue. After La^{3+} removal from the apical bathing fluid (Fig. 8), tissues respond to the withdrawal of basolateral Ca^{2+} as a fresh tissue. Conversely, the addition of La^{3+} (2 μM) to the apical solution after the permeability of the TJs had been increased in response to basolateral Ca^{2+} removal has two distinct effects (Fig. 8 *B*): (*a*) apical La^{3+} promptly terminates the process of TER decrease, and (*b*) its presence in the apical compartment blocks TER recovery that takes place in response to basolateral Ca^{2+} addition. The first effect resembles that of Ca^{2+}, Cd^{2+}, and Mn^{2+}. The second indicates that open TJs permit La^{3+} to enter from the apical side and reach the Ca^{2+} binding sites that control TJ function, acting as if La^{3+} had been added to the basolateral fluid.

DISCUSSION

The present study deals with the interactions of metal ions with the extracellular Ca^{2+}-binding sites that modulate the TJs in the frog urinary bladder. Focus was addressed to the early events associated with TJ opening and closing.

The dependence of TER, which reflects the degree of permeability of the TJs on basolateral Ca^{2+} concentration was evaluated to characterize the dependence of the TJ regulatory system of the frog urinary bladder on the external Ca^{2+} concentration. The steep dependence of TER on $[Ca^{2+}]_{bl}$, with a K_m of 62 ± 28 μM and a Hill coefficient of 8.6 ± 0.9 (n = 5), indicates a high Ca^{2+} affinity of the extracellular Ca^{2+} sites and is in agreement with other tissues, such as MDCK (González-Mariscal et al., 1990) and A6 (Jovov et al., 1994) cell monolayers.

The results with the intracellular Ca^{2+} chelator indicates that a rise of intracellular Ca^{2+} concentration is not a critical step in the resealing of TJs induced by

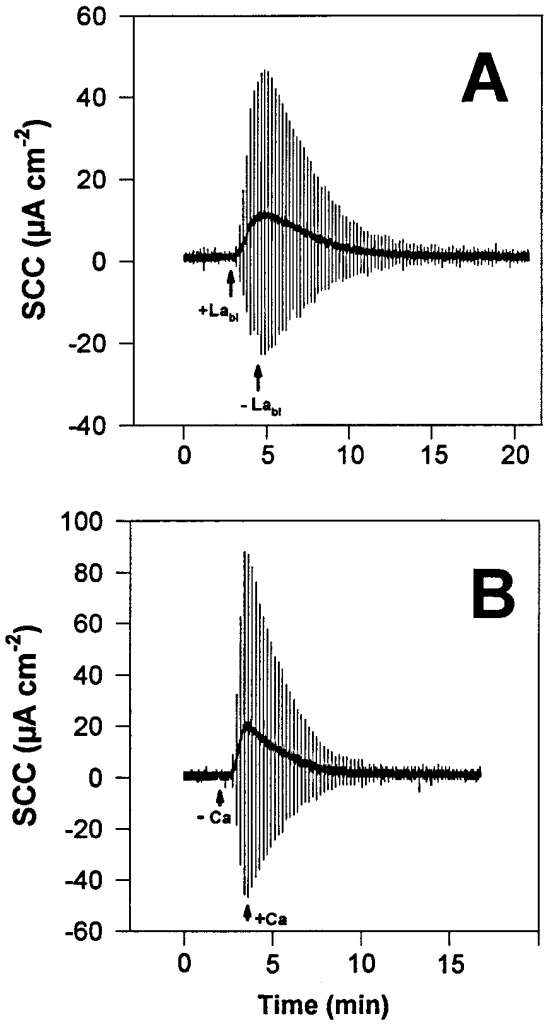

FIGURE 7. Representative experiment (of a group of nine tissues) of the action of basolateral La^{3+} (2 μM as $La(NO_3)_3$) on the TJ seal, as evaluated by TER. The urinary bladders were short-circuited and bathed on the apical side by 75 mM KCl and on the basolateral side by NaCl HEPES Ringer's solution. (*A*) TER decreases in response to addition of basolateral La^{3+} ($+La_{bl}$) and fully recovers upon La^{3+} removal ($-La_{bl}$). (*B*) Control experiment performed in the same tissue of the effect of basolateral Ca^{2+} withdrawal and subsequent reintroduction, showing the similarity between the responses to La^{3+} addition and Ca^{2+} removal. The vertical deflections of SCC, caused by pulses of ±1 mV in the clamping potential, are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

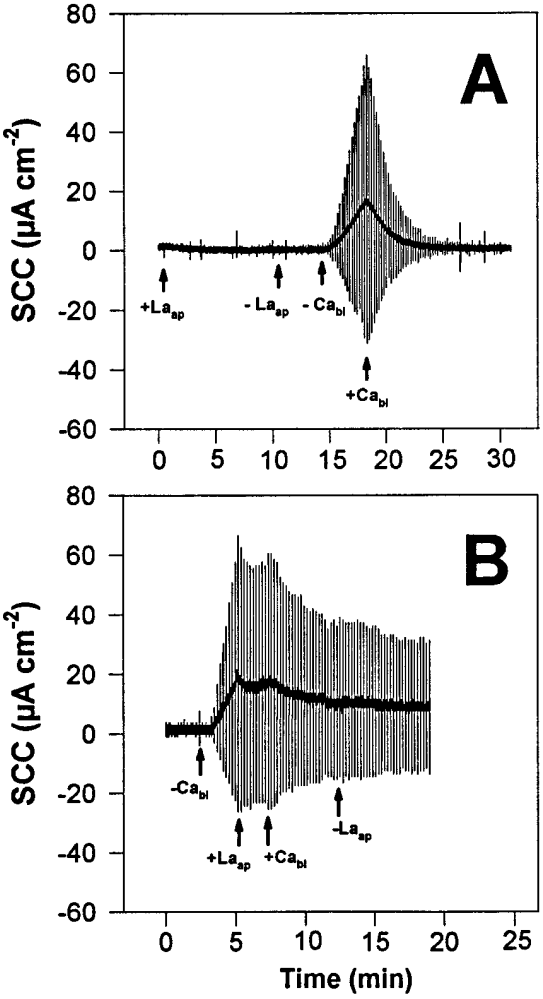

FIGURE 8. Representative experiments (in two groups of six tissues) of the action of apical La^{3+} on tissue response to manipulation of basolateral Ca^{2+}. (*A*) Addition of La^{3+} (2 μM as $La(NO_3)_3$) to the apical solution ($+La_{ap}$) has no effect whatever upon TER. Its subsequent removal ($-La_{ap}$) renders the tissue responsive to the removal ($-Ca_{bl}$) and reintroduction ($+Ca_{bl}$) of basolateral Ca^{2+}, as in control tissues not exposed to apical La^{3+}. (*B*) Addition of La^{3+} (2 μM as $La(NO_3)_3$) to the apical solution ($+La_{ap}$) during TER decrease caused by removal of basolateral Ca^{2+} ($-Ca_{bl}$) terminates the process of TER decrease and blocks TER recovery induced by reintroduction of basolateral Ca^{2+} ($+Ca_{bl}$). The vertical deflections of the SCC, caused by pulses of ±10 mV in the clamping potential, are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

raising the basolateral Ca^{2+} concentration. Nonetheless, it cannot be ruled out that a sudden increase of cytosolic Ca^{2+} concentration may activate, at least transiently, the mechanism of TJ sealing. However, a complete and stable recovery of the TJ seal was only obtained upon addition of Ca^{2+} to the basolateral solution. Our findings are in agreement with observations in monolayers of human cervical cell line CaSki, where the effects of extracellular Ca^{2+} on TJ permeability were found not to be mediated by mobilization of cytosolic Ca^{2+} (Wild et al., 1997). On the other hand, the observations with the Ca^{2+} ionophore recalls the findings in A6 cell monolayers, where an increase of cytosolic Ca^{2+} concentration induced by the ionophore A-23187 caused recovery of the TJ seal (Jovov et al., 1994).

The result of a prolonged extracellular Ca^{2+} withdrawal has been described as causing a progressive disarray of the TJ structure in natural epithelia or in cell-cultured monolayers. In short-term experiments,

however, Ca^{2+} removal is not accompanied by gross distortions of freeze-fracture images (Martinez-Palomo and Erlij, 1975; Martinez-Palomo et al., 1980; Lacaz-Vieira and Kachar, 1996), indicating that the rapid phase of TER drop after Ca^{2+} withdrawal might result from subtle alterations of the TJs structure not detectable by conventional methods.

The finding that the alkaline earth metals, Mg^{2+} and Ba^{2+}, used as controls, were ineffective both in keeping the TJs closed and inducing the resealing of previously opened TJs is in consonance with findings in MDCK cell monolayers where Mg^{2+} and Ba^{2+} were also ineffective in promoting junction resealing (Martinez-Palomo et al., 1980; Contreras et al., 1992).

The transition elements, Mn^{2+} and Cd^{2+}, behave as Ca^{2+} agonists since, in the absence of basolateral Ca^{2+}, they promote stability of the TJs and halt almost instantly the TJ opening process triggered by basolateral Ca^{2+} removal. These effects of Mn^{2+} and Cd^{2+}, which resemble the action of Ca^{2+}, might result from their interaction with E-cadherin molecules that are mostly concentrated in the zonula adhaerens (Boller et al., 1985) and are known to be involved in the assembly of the junctional complex (Gumbiner et al., 1988). In addition, Mn^{2+} promotes an almost complete recovery of TER, closely resembling the effect of extracellular Ca^{2+}. This effect of Mn^{2+} is in harmony with the observation in the toad urinary bladder, where Mn^{2+} and Sr^{2+} were able to revert the rapid fall of TER that takes place when Ca^{2+} was withdrawn from the medium (Lipson et al., 1965). In contrast, in the bullfrog gastric mucosa, Sr^{2+} does not promote recovery of the junctional seal (Forte and Nauss, 1963); in MDCK cell monolayers, only Ca^{2+} was effective in triggering TJ formation during a Ca^{2+} switch; Mg^{2+}, Ba^{2+}, Mn^{2+}, and Cd^{2+} were ineffective (Contreras et al., 1992). These results show that major differences can be found among tissues and, for the sake of consistency, one given tissue must be thoroughly studied.

E-cadherin, in addition to its affinity for Ca^{2+} (Ringwald et al., 1987), also binds Cd^{2+}, as can be inferred from binding experiments with E-CAD1, a recombinant 145-residue polypeptide that corresponds to one of the extracellular Ca^{2+}-binding regions of E-cadherin (Prozialeck et al., 1996). In contrast to our experiments, where Cd^{2+} stabilizes the TJs in the closed state and interrupts the TJ opening process that is triggered by basolateral Ca^{2+} removal, recent studies show that Cd^{2+} can selectively damage the TJs between LLC-PK1 cells (Prozialeck et al., 1995) and human proximal tubule cells (Hazen Martin et al., 1993) and causes disruption of the TJ-associated microfilaments in rat Sertoli cells (Hew et al., 1993). It is conceivable that this discrepancy might result from the duration of Cd^{2+} contact with the preparation. In our case, brief tissue exposures to Cd^{2+} might have prevented the onset of major toxic effect, which might have been the cause of TJ disruption in other structures. Consequently, our short-term experiments apparently permit us to dissociate an agonistic effect of Cd^{2+} on the TJs from a less specific toxic effect.

How could Cd^{2+} stabilize the TJs in closed state, halt the TJ opening process but, at the same time, be unable to promote recovery to the TJ seal? A reasonable interpretation would be that Cd^{2+} acts as a Ca^{2+} agonist but, in addition, it presents toxic side effects that develop at a slower pace, preventing the recovery of the TJ seal. The possibility cannot be discarded that Cd^{2+} (as well as Ca^{2+} and Mn^{2+}), in addition to interacting with E-cadherin, acts by bridging junctional sites at the level of the TJs themselves. This interpretation is supported by experiments in MDCK cell monolayers prefixed with glutaraldehyde, where Ca^{2+} removal still caused a pronounced drop of TER (Martinez-Palomo et al., 1980). In disharmony, however, is the observation that in junctional complex–enriched fractions from mouse liver, Ca^{2+} chelation with EGTA does not disrupt the negative-stained images of zonulae occludentes (Stevenson and Goodenough, 1984).

The fact that Cd^{2+} does not leave a residual, apparently toxic, effect when its contact with the tissue takes place in the presence of Ca^{2+} may result from a competitive interaction with Ca^{2+} for a common binding site, which most probably is E-cadherin. In support of this interpretation are the observations in LLC-PK1 cell monolayers that Cd^{2+} shows a higher binding affinity at low (0.1 mM) than at high (10 mM) Ca^{2+} concentrations (Prozialeck and Lamar, 1993), and also the experiments of Cd^{2+} binding to E-CAD1, a Ca^{2+} binding polypeptide analog of E-cadherin (Prozialeck et al., 1996).

Previous studies in frog skin (Castro et al., 1993) and urinary bladder (Lacaz-Vieira and Kachar, 1996) have shown that apical Ca^{2+} may reach the sites that control the TJs when the permeability of the TJs was increased. As Ca^{2+} channel blockers in the apical solution did not block the effect of apical Ca^{2+} (Lacaz-Vieira and Kachar, 1996), it can be inferred that apical Ca^{2+}, passing through partially opened TJs, may reach the sites that affect the TJs located at the zonula adhaerens. In the present study, we explored in more detail this subject and showed that at concentrations higher than those of the Ringer's solution, apical Ca^{2+} is able to effectively fulfill the role of basolateral Ca^{2+}, maintaining the TJs closed or even causing the resealing of open TJs in the absence of basolateral Ca^{2+}. The fact that to be effective apical Ca^{2+} concentrations must be higher than that needed in the basolateral solution is reasonable if we take into consideration the diffusion barrier imposed by the TJs before Ca^{2+} reaches the zonula adhaerens.

The fact of Mg^{2+} competitively inhibiting the Ca^{2+}-induced recovery of open TJs means that Mg^{2+} interacts with the Ca^{2+} binding sites, despite the fact that this interaction, in the absence of Ca^{2+} has no effect whatsoever on the stability of the TJs, the halting of the opening process, or recovery of the TJ seal. This competitive inhibition is in agreement with findings in A6 cell monolayers (Jovov et al., 1994).

Another aspect of the interaction of Mg^{2+} with the TJ regulatory system is the dissociation between the ability of basolateral Ca^{2+} to stop almost instantly the process of TJ opening, which is preserved, and the ability to induce the recovery of TER, which is abolished by Mg^{2+}. This dualistic behavior may be regarded as an argument in favor of a dual effect of Ca^{2+}. The preserved effect could be due to the formation of ionic bridges between components in adjacent cells or, most likely, Ca^{2+}-induced changes in E-cadherin adhesiveness (Ringwald et al., 1987). The other, slower, abolished by Mg^{2+}, is the recovery of the TJ seal, presumably resulting from a rearrangement of the TJ molecular organization mediated by cell signaling triggered by the interaction of Ca^{2+} with E-cadherin.

The fact that 200 μM La^{3+} causes a reversible TJ opening in the presence of 1 mM Ca^{2+} characterizes an antagonistic effect for Ca^{2+} in an apparent competitive interaction. Interaction of La^{3+} with Ca^{2+} binding sites has been studied in different structures (Weiss, 1974). La^{3+} is a modulator of gating activity of ionic channels (Takata et al., 1966; Vogel, 1974; Hille et al., 1975; Armstrong and Cota, 1990; Watkins and Mathie, 1994), a potent Ca^{2+} channel blocker (Nelson, 1987; Poncet et al., 1992; Clarke et al., 1994), and may also act as a Ca^{2+} agonist (Powis et al., 1994). These effects may result from the fact that La^{3+}, by virtue of an effective ionic radius (1.10 Å) similar to that of Ca^{2+} (1.06 Å) (Snyder et al., 1990) and a valence higher than Ca^{2+}, is expected to bind at Ca^{2+} sites more tightly than does Ca^{2+}. The action of La^{3+} on the TJs is a complicated matter since different effects have been described. Our present finding that La^{3+} promotes TJ opening in the presence of Ca^{2+} contrasts with those in MDCK cells, where La^{3+} was used as a Ca^{2+} channel blocker and found not to interfere with junction sealing (Contreras et al., 1992), with neurophysiological experiments (where La^{3+} was used as a TJ blocker) (Sostman and Simon, 1991; Simon, 1992; Bryant and Moore, 1995; Wang et al., 1993), and with experiments in A6 cell monolayers, where a toxic effect was reported (Jovov et al., 1994). In the frog skin, however, the reversible opening of the TJs by an apical hypertonicity (Ussing, 1965) was made irreversible by the presence of La^{3+} in the apical solution (Erlij and Martinez-Palomo, 1972), indicating that La^{3+} interacted with open TJs, preventing their resealing. Transient effects of La^{3+} on TER and changes of ion selectivity have been described in rabbit gallbladder and ileum (Machen et al., 1972). Studies of protein Ca^{2+} sites have indicated that La^{3+} is often able to effectively replace bound Ca^{2+} because of the proximity of their effective ionic radius and because many Ca^{2+} sites bind both divalent and trivalent metal ions with high affinity (Brittain et al., 1976; Horrocks, 1984). Comparison of the dissociation constants for the binding of spherical metal ions from groups IA, IIA, IIIA, and lantanides indicates that both charge and size are important parameters in determining the specificity of the protein binding sites (Snyder et al., 1990).

The ability of La^{3+} to open TJs in the presence of Ca^{2+} is a relevant matter to the general use of La^{3+} in the evaluation of the permeability of TJs in epithelial and endothelial membranes (Arendt, 1991; Unakar et al., 1991; Vu et al., 1992; Shirai and Ikemoto, 1992;

TABLE I

Comparative Effects of Metal Ions on the Dynamics of TJs, as Observed in the Present Study, and on the Uvomorulin Molecule (Hyafil et al., 1981)

TJs	Uvomorulin
• Ca^{2+}, Mn^{2+}, and Cd^{2+} confer stability to the TJs, but not to Mg^{2+} or Ba^{2+}.	• Ca^{2+}, Mn^{2+}, and Cd^{2+} confer trypsin resistance to Umt (an 84-kD fragment of uvomorulin) and trigger the recognition of Umt by a monoclonal antibody. Mg^{2+} and Ba^{2+} are ineffective.
• Ca^{2+} and Mn^{2+} are able to trigger the resealing of TJs previously opened by Ca^{2+} removal. Cd^{2+} is ineffective and apparently has a toxic effect upon the epithelial cells. Mg^{2+} and Ba^{2+} were ineffective.	• Ca^{2+} and Mn^{2+} (but not Cd^{2+}, which is toxic to cells) prevent EC cells or morulae from decompacting in Ca^{2+}-free medium. Mg^{2+} and Ba^{2+} were ineffective.
• La^{3+} did not confer stability to the TJs. In addition, La^{3+} in the presence of Ca^{2+} reverts the Ca^{2+}-dependent stability of the TJs, thus behaving as an inhibitor of the Ca^{2+} action.	• La^{3+} did not protect Umt against trypsin digestion. In addition, La^{3+} in the presence of Ca^{2+} reverts the Ca^{2+}-induced protection, thus behaving as an inhibitor of Ca^{2+} action.
• The inhibitory action of La^{3+} upon the Ca^{2+} effect is reversible upon La^{3+} removal.	• The inhibitory action of La^{3+} upon the Ca^{2+} effect is reversible upon La^{3+} removal.

Caldwell and Slapnick, 1992; Morales and Cavicchia, 1993; Adamson and Michel, 1993; Zhong et al., 1994; Pelletier, 1994; Hochman et al., 1994; Hara et al., 1994; Devalia et al., 1994), particularly during in vivo perfusions or in the absence of a simultaneously present fixative such as glutaraldehyde or formaldehyde (Martinez-Palomo et al., 1971; Whittembury and Rawlins, 1971; Machen et al., 1972; Martinez-Palomo and Erlij, 1973; Tisher and Yarger, 1973). A comparative study of the permeability of TJs of blood barriers of the epididymis, vas deferens, and testis in the mink, using horseradish peroxidase and lanthanum nitrate, showing that lanthanum deposits were found at the microvilli despite the impermeability of the TJs to horseradish peroxidase, permitted the authors to suggest that the lanthanum technique yielded false positive results (Pelletier, 1994). It is conceivable that in this case some TJs could have been opened by the effect of La^{3+} before the action of fixatives could have taken place. Supporting this interpretation are studies (with high resolution electron micrographs of TJs in different structures in which La^{3+} was used during fixation) that have given no evidence that these junctions were permeable to colloidal lanthanum (Overton, 1968; Brightman and Reese, 1969; Goodenough and Revel, 1970), in contrast to experiments in which La^{3+} was perfused in living tissue (Schatzki, 1969, 1971), where there are evidences of lanthanum passage through the TJs.

To conclude, it is rewarding to compare (Table I) the effects of Ca^{2+} and metal ions upon uvomorulin (E-cadherin) in a study of early embryogenesis (Hyafil et al., 1981), where the authors concluded that uvomorulin undergoes a Ca^{2+} (or Mn^{2+}- or Cd^{2+}-)-dependent transition from a trypsin-sensitive to a trypsin-resistant conformation that favors recognition of uvomorulin by a monoclonal antibody and triggers cell compaction in early embryogenesis, and the findings of the present study. The close similarity of behavior observed in those two systems in response to similar treatments is a strong indication that the modulation Ca^{2+} and other metal ions exert on the TJs is most importantly mediated through their interaction with E-cadherin molecules.

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