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Lanthanum Effect on the Dynamics of Tight Junction Opening and Closing

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Abstract. We present a comparative study in frog urinary bladders (FUB) and A6 cell monolayers (A6CM) on the effect of La^{3+} on tight junction (TJ) dynamics. These tissues react similarly to changes of basolateral Ca^{2+} ($Ca^{2+}{}_{bl}$), while responding differently to the action of $La^{3+}{}_{bl}$. In FUB, $La^{3+}{}_{bl}$ shows a Ca^{2+}-antagonistic effect that promotes TJ opening in the presence of a normal $Ca^{2+}{}_{bl}$ concentration. In A6CM, in contrast, $La^{3+}{}_{bl}$ always shows a clear Ca^{2+}-agonistic effect. The fact that a concentration of $La^{3+}{}_{bl}$ one fifth of the normal $Ca^{2+}{}_{bl}$ leads in FUB to TJ opening and in A6CM to a complete recovery of the TJ seal indicates a high affinity of La^{3+} for the Ca^{2+}-binding sites in both tissues. In FUB, apical La^{3+} ($La^{3+}{}_{ap}$) exhibits, differently from its basolateral effect, an evident Ca^{2+}-agonistic effect, suggesting a dual effect of La^{3+}, depending on which side of the bladder La^{3+} is applied. In A6CM $La^{3+}{}_{ap}$ has a Ca^{2+}-agonistic effect similar to $La^{3+}{}_{bl}$. The effects of $La^{3+}{}_{bl}$ in FUB and in A6CM are consistent, according to our previous publications, with La^{3+} acting antagonistically or agonistically, respectively, on the Ca^{2+} binding sites of *zonula adhaerens*. Despite the fact that the effect of $La^{3+}{}_{ap}$ is clear in both tissues, its site of action is yet to be determined. Protonation of the Ca^{2+}-binding sites causes a decrease of its agonistic effect on A6CM, consistent with a negatively charged binding site. In A6CM La^{3+} apparently replaces Ca^{2+}, mimicking the effect of Ca^{2+} triggering the cascade of events leading to TJ closure. In FUB, La^{3+} interacts with the binding sites, dislodging Ca^{2+}, with a high affinity, but this interaction is inadequate to initiate or sustain the process of junction closing. Possibly, the difference between the two preparations resides in subtle conformation differences of the outer segment of E-cadherin molecules.

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Key words: Calcium — Lanthanum — Tight junction — E-cadherin — A6 cells — Frog urinary bladders

Introduction

Epithelial membranes are polarized structures with cells held together by the junctional complex, the tight junction (TJ) being its most apical component. TJs form a morphological and functional, highly dynamic boundary between the apical and basolateral cell surface domains and regulate transport along the paracellular route. For reviews, *see* Cereijido et al. (1988, 1989), Balda et al. (1992), Cereijido et al. (1998), Anderson and Van Itallie (1999), Brown and Davis (2002), Gonzalez-Mariscal et al. (2003), Schneeberger and Lynch (2004).

Extracellular Ca^{2+} is long known to be essential for the formation and stability of the TJs in natural epithelia (Sedar & Forte, 1964; Hays, Singer & Malamed, 1965; Galli, Camilli & Meldolesi, 1976; Meldolesi et al., 1978; Palant et al., 1983; Pitelka, Taggart & Hamamoto, 1983) and in cell culture monolayers in confluence (Cereijido et al., 1980; Martinez-Palomo et al., 1980; Cereijido, Meza & Martinez-Palomo, 1981; González-Mariscal, Chávez de Ramirez & Cereijido, 1985). Ca^{2+} interacts with extracellular binding sites, the cell adhesion molecule E-cadherin (Gumbiner, Stevenson & Grimaldi, 1988), mostly located at the *zonula adhaerens* (Boller, Vestweber & Kemler, 1985). Compelling evidences (Shapiro et al., 1995; Nagar et al., 1996; Tomschy et al., 1996; Yap et al., 1997) indicate that the process of cadherin-mediated adhesion consists of a parallel, lateral *cis*-interaction and an antiparallel, adhesive *trans*-interaction, both Ca^{2+}-dependent processes with different binding affinities (Koch et al., 1997). Ca^{2+}-induced cadherin adhesion triggers a cascade of events responsible for the stability of TJs as well as

for the assembly of new TJs in recently plated cell monolayers (Balda et al., 1991, 1993).

The extracellular Ca^{2+}-binding sites interact not only with Ca^{2+}, but metal ions can bind and affect TJ dynamics. In the frog urinary bladders it was shown (Lacaz-Vieira, 1997) that Mg^{2+} and Ba^{2+} were incapable of keeping the TJ sealed or of inducing TJ recovery; Mg^{2+} caused a reversible concentration-dependent inhibition of the Ca^{2+}-induced TJ recovery; the transition elements Mn^{2+} and Cd^{2+} acted as Ca^{2+} agonists and La^{3+}, which presented a high affinity for the Ca^{2+}-binding sites, caused an increase of TJ permeability that fully reverses after its withdrawal (Lacaz-Vieira, 1997). It was pointed out that the ability of La^{3+} to open TJs in the presence of extracellular Ca^{2+} is an indication that La^{3+} dislodges Ca^{2+} from its binding sites with high affinity. This observation is a relevant aspect that must be considered when interpreting results of experiments using La^{3+} for assessing the paracellular permeability of epithelial and endothelial membranes. The effect of La^{3+} in promoting increase of TJ permeability is of major concern when this ion is used as a paracellular tracer (Ramsey, Bernd & Knox, 1998, 2002) or as an electron-dense paracellular marker in electron microscopy, particularly when tissues are submitted to in vivo perfusion with solutions containing La^{3+} (Machen, Erlij & Wooding, 1972; Alvarado, Dietz & Mullen, 1975; Ramsey et al., 1998; Gleeson et al., 2000) or even solutions containing La^{3+} associated with fixatives (Lora et al., 1997; Uehara & Miyoshi, 1999; Lippoldt et al., 2000; Mazzon et al., 2002; Suzuki et al., 2002; Weiss et al., 2003). The effect of La^{3+} varies in different tissues: in some cases a Ca^{2+}-agonistic effect is seen; in others an antagonistic outcome is present. Thus, La^{3+} is a modulator of gating activity of ionic channels (Takata et al., 1966; Vogel, 1974; Hille, Woodhull & Shapiro, 1975; Armstrong & Cota, 1990; Watkins & Mathie, 1994), a Ca^{2+}-channel blocker (Nelson, 1987; Poncet, Merot & Poujeol, 1992; Clarke, Moore & Blalock, 1994). Less frequently, La^{3+} may show a Ca^{2+}-agonistic effect (Powis, Clark & O'Brien, 1994). La^{3+} exerts positive modulation on muscle nicotinic acetylcholine receptors (AChRs), whereas it modulates negatively neuronal AChRs (García-Colunga & Miledi, 1997). In MDCK cells, La^{3+} and Gd^{3+} were able to block capacitative Ca^{2+} entry (Jan et al., 1999).

The high affinity of La^{3+} for the Ca^{2+} sites might result from the combined effect of two factors, an effective radius (1.10 Å) similar to that of Ca^{2+} (1.06 Å) (Snyder, Buoscio & Falke, 1990) and a valence higher than that of Ca^{2+}.

In the present work we have extended our previous observations on the effect of La^{3+} in frog urinary bladders (Lacaz-Vieira, 1997) by performing a comparative study using two distinct tissues that respond differently upon La^{3+} challenge.

Materials and Methods

Two preparations were used in the present study: A6 cell monolayers and excised frog urinary bladders.

Cell Culture

A6 cells (CCL 102) obtained from American Type Culture Collection (Rockville, MD) were grown at room temperature in CL2-Amphibian medium (NIH-Media Section, Bethesda, MD), 10% fetal bovine serum (Sigma Chemical, St. Louis, MO) and 2 mM glutamine Pen-Strepto (Sigma). Cells at confluence were harvested with 0.25% trypsin solution (Sigma). The cell suspensions were plated, at a density high enough to reach confluence in several hours, on 6-well plates with Transwell cell culture inserts (Transwell COL, collagen-treated filters containing a mixture of collagen types I and III; 4.7 cm^2 growth area and 0.4 μm pore size; Costar, Cambridge, MA). Confluent monolayers reached a stable electrical conductance (G) averaging $4.21 \times 10^{-4} \pm 3.3 \times 10^{-5}$ S/cm^2 ($n = 37$) around day 14. Monolayers 14 to 18 days old were used in the experiments.

Plastic rings of 20 mm diameter were glued with ethylcyanoacrylate adhesive (Super Bonder, Loctite) to the side of the support filters opposite to where the cells were attached. The monolayer fragment framed by the plastic ring was excised and immersed in Ringer solution.

Urinary Bladders

Urinary bladders of the frog *Rana catesbeiana* were obtained from animals anesthetized by subcutaneous injection of a 2% solution of 3-aminobenzoic acid ethyl ester (methanesulfonate salt) (Sigma) at a dose of 1 ml/100 g of body weight. The abdominal cavity was opened, a cannula was passed through the cloaca and the urinary bladder was inflated with 15 to 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 (Pronto CA8, 3M or Super Bonder, Loctite). The fragment of tissue framed by the plastic ring was excised and immersed in Ringer solution.

Procedures

Excised bladder fragments or cultured cell monolayers were subsequently mounted in a modified Ussing's chamber (Castro, Sesso & Lecaz-Vieira, 1993), exposing an area of 0.5 cm^2. Hemichambers with a recessed rim filled with high viscosity silicone grease (Dow Corning High Vacuum Grease) 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 basolateral bathing solution was NaCl Ringer's solution with the following composition (in mM) for NaCl-Ringer: NaCl 115, $KHCO_3$ 2.5, and $CaCl_2$ 1.0, for NaCl HEPES Ringer: NaCl 115, KCl 2.5, HEPES 2.0. Unless otherwise indi-

cated, the Ringer's solutions had pH adjusted to 8.2. The apical bathing fluids were simple salt solutions, non-buffered, prepared with glass-distilled water, having pH around 6.0 and free Ca^{2+} concentration in the range of 1.5×10^{-7} and 2.0×10^{-7} M (Castro et al., 1993). In the beginning of the experiment the apical solution was KCl 75 mM to eliminate Na^+ from this solution, in order to rule out the contribution of transcellular Na^+ conductance to the overall tissue electrical conductance. No EGTA was used in the bathing solutions since this chelating agent diffusing into the lateral spaces affects the time course of Ca^{2+} concentration increase or decrease in this region in response to changes of Ca^{2+} concentration in the bathing solutions. In the figures, all concentrations are expressed in mM.

Electrical Measurements

A conventional analog voltage clamp (WPI DVC 1000) was used. Saturated calomel half-cells with 3 M KCl-agar bridges were used to measure the electrical potential difference across the skin. 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 skin. The clamping current was continuously recorded by a strip-chart recorder. Clamping current and voltage were also digitized through an analog-to-digital converter at a digitizing rate of 100 Hz (Digidata 1200 and Axotape 2.0, Axon Instruments, Inc.) and stored in a computer for further processing.

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO). La^{3+} was used as a nitrate salt.

Statistics

The results are presented as mean ± standard error of the mean. Comparisons were carried out using Student's paired *t*-test, (Neter & Wasserman, 1974).

Fast Ca^{2+}-Switch Assay (FCSA)

Tissues were bathed in nominally Ca^{2+}-free apical solution. The TJs were opened by removal of Ca^{2+} from the basolateral solution, inducing an increase of the overall tissue electrical conductance (*G*). Subsequent resealing of the TJs was induced by reintroducing Ca^{2+} into the basolateral fluid, causing a decrease of *G* towards initial control levels. The action of drugs on the TJs was tested by studying their effects on the dynamics of TJ opening and closing in response to the FCSA.

Abbreviations and Conventions

TJ: tight junction
FCSA: fast Ca^{2+}-switch assay.
I: Clamping current, in μA cm^{-2}. Positive current corresponds to the transport of positive charges across the bladder from the apical to the basolateral solution.
V: Electrical potential difference across the bladder, in mV. The potential of the apical solution is referred to that of the basolateral solution.
G: transepithelial electrical conductance, in S/cm^2. *G* was calculated using a data analysis and technical graphing software Origin™ (version 5) (Microcal Software). The clamping current was initially smoothed by an adjacent-points averaging procedure (200 points) to obtain the short-circuit current (*SCC*). *SCC* was then subtracted from the clamping current to remove offset and then the peak current values were calculated and from these, the transmembrane electrical conductance was obtained by Ohm's law.

Results

The present experiments aimed to analyze the interactions of La^{3+} with the extracellular Ca^{2+} binding sites of *zonula adhaerens*, sites that modulate the dynamics of the TJs. Two different preparations were used, isolated frog urinary bladders and monolayers of renal cells in culture (A6 cell monolayers). These tissues show distinct TJ behaviors in many experimental conditions to which they are submitted, as in response to inhibitors of PKC (Lacaz-Vieira, 2000; Lacaz-Vieira & Jaeger, 2001) or regarding the existence of TJ permeability oscillations when submitted to $Ca^{2+}{}_{ap}$ in an FCSA (Kassab, Jr., Marques & Lacaz-Vieira, 2002).

Results From Frog Urinary Bladder

La^{3+} addition to normal Ca^{2+}-containing (1 mM) Ringer's solution bathing the basolateral tissue surface starts to have an effect from concentrations as low as 0.01 mM. The responses to La^{3+} of frog urinary bladders are always characterized by an increase of TJ permeability, as indicated by an increase of *G*, a reliable index of paracellular permeability (Lacaz-Vieira & Kachar, 1996). At low $La^{3+}{}_{bl}$ concentrations (0.02 mM), the increase of *G* is small, and a plateau level is soon attained (Fig. 1*A*). Higher concentrations (0.06 mM) trigger a conspicuous response characterized by a marked increase of *G*, which is interrupted and reverses after $La^{3+}{}_{bl}$ withdrawal (Fig. 1*A*). This response resembles closely that observed when $Ca^{2+}{}_{bl}$ is, respectively, removed and subsequently replaced, as shown for a representative FCSA experiment (Fig. 1*B*). Differently from an FCSA, however, where $Ca^{2+}{}_{bl}$ reintroduction leads to an abrupt halt of *G* increase immediately followed by a recovery process, in the La^{3+} experiments tissue recovery triggered by La^{3+} withdrawal is always preceded by a delay, which gets longer with increase in La^{3+} concentration and/or La^{3+} exposure time. Figure 2 is a representative example of the effect of a short-duration higher concentration $La^{3+}{}_{bl}$ pulse (0.2 mM), where the delay preceding the start of the recovery phase is clearly observed.

Differently from its basolateral effect, where La^{3+} always leads to an increase of *G*, when tested in the apical solution, La^{3+} has a different effect. For example, if La^{3+} is added to the apical bathing fluid while *G* is increasing due to $Ca^{2+}{}_{bl}$ removal, it slows down the rate of *G* increase (Fig. 3*A*). If $La^{3+}{}_{ap}$ is

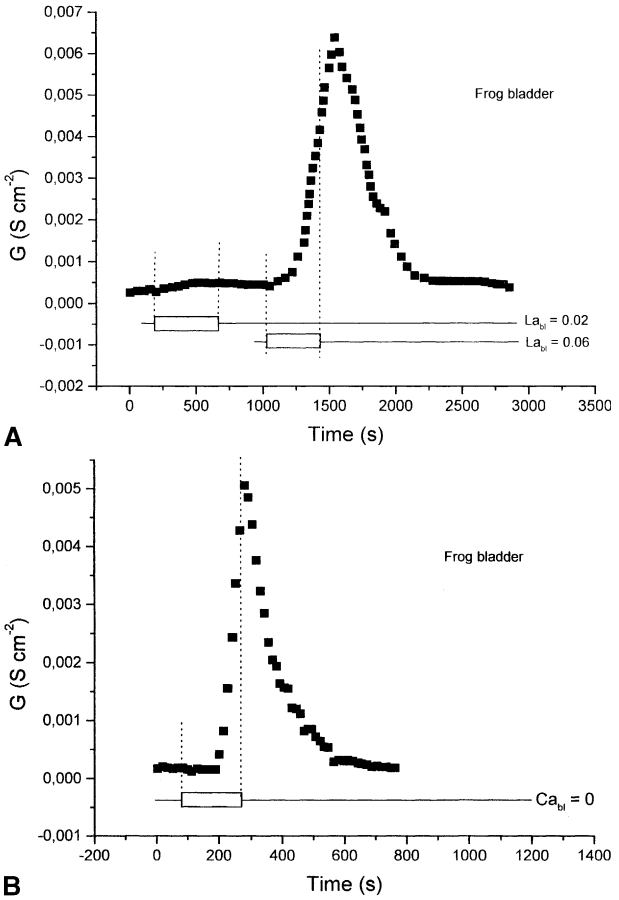

Fig. 1. (*A*) Representative experiment in frog urinary bladder (of a group of 8) on the action of $La^{3+}{}_{bl}$ (0.02 mM) added to the basolateral solution followed by its withdrawal after 500 s. At this lower concentration basolateral La^{3+} has a small effect on tissue electrical conductance (*G*). Later, a second pulse of $La^{3+}{}_{bl}$ of 0.056 mM and same duration was applied to the basolateral solution. The response is characterized by a fast increase of *G*, which continues to increase for a short time after La^{3+} removal and then slowly recovers completely. The apical solution was KCl 75 mM and basolateral solution, NaCl HEPES Ringer. In this and all subsequent figures, the rectangles indicate the period in which the concentrations are those specified at the right and expressed in mM. (*B*) Representative experiment (of a group of 5) in frog urinary bladder on the dynamics of TJ opening and closing in a fast Ca^{2+}-switch assay (FCSA) in which $Ca^{2+}{}_{bl}$ is removed and subsequently returned, causing an increase and then recovery of *G*. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

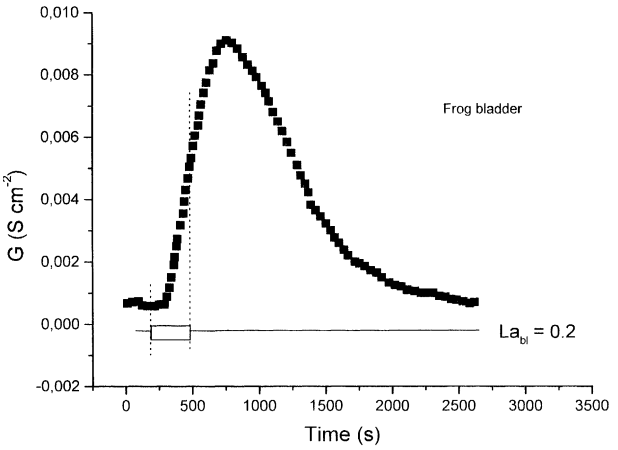

Fig. 2. Representative experiment in frog urinary bladder (of a group of 6) on the action of La^{3+} (0.2 mM) added to the basolateral solution followed by its withdrawal after 300 s. The response is characterized by a fast increase of *G*, which continues to increase after La^{3+} removal and then slowly recovers completely. The apical solution was KCl 75 mM and the basolateral solution NaCl HEPES Ringer.

already present, removal of $Ca^{2+}{}_{bl}$ brings about a much slower rate of *G* increase (Fig. 3*B*) as compared to a control experiment in the absence of La^{3+} (Fig. 1*B*). These different results, according to which side of the tissue La^{3+} is tested, might imply a dual effect of La^{3+}. These experiments (Fig. 3*A* and 3*B*) also show that apical La^{3+}, at a concentration that clearly causes TJ opening when present in the basolateral solution (0.2 mM) (Fig. 2), is unable to bring about TJ opening, since a rapid, almost full recovery takes place when Ca^{2+} is returned to the basolateral solution. In favor of the dual-effect interpretation, depending on the side of tissue La^{3+} is tested, is the result shown in Fig. 4, in which La^{3+} was initially added to the basolateral bathing solution in the presence of $Ca^{2+}{}_{bl}$, causing an increase of TJ permeability that did not reverse quickly upon La^{3+} removal, due to the high concentration of and long tissue exposure to La^{3+}. A subsequent addition of La^{3+} to the apical solution at the same concentration (0.2 mM) triggers a marked decline of *G*, which was promptly interrupted and reversed when La^{3+} was also added to the basolateral solution.

When La^{3+} is added to the basolateral Ringer's solution at a concentration equal to that of Ca^{2+} (1 mM), we observe an increase of *G*, as shown previously. After La^{3+} withdrawal, due to its large concentration, no recovery takes place within a reasonable time interval. A subsequent addition of a high Ca^{2+} concentration to the apical bathing fluid triggers a recovery process that keeps going even after $Ca^{2+}{}_{ap}$ is removed. A representative experiment is shown in Fig. 5. This result supports the interpretation that a high concentration of $Ca^{2+}{}_{ap}$ drives Ca^{2+} into open TJs, dislodging La^{3+} from the binding sites of *zonula adhaerens*, thus enabling tissue recovery to continue when Ca^{2+} concentration is returned to normal control levels.

In order to better characterize the dual effect of La^{3+}, depending on the side of tissue La^{3+} is added, we performed experiments in which TJs were opened by $Ca^{2+}{}_{bl}$ removal and subsequently La^{3+} was added to the apical bathing solution. A representative experiment is shown in Fig. 6, where it

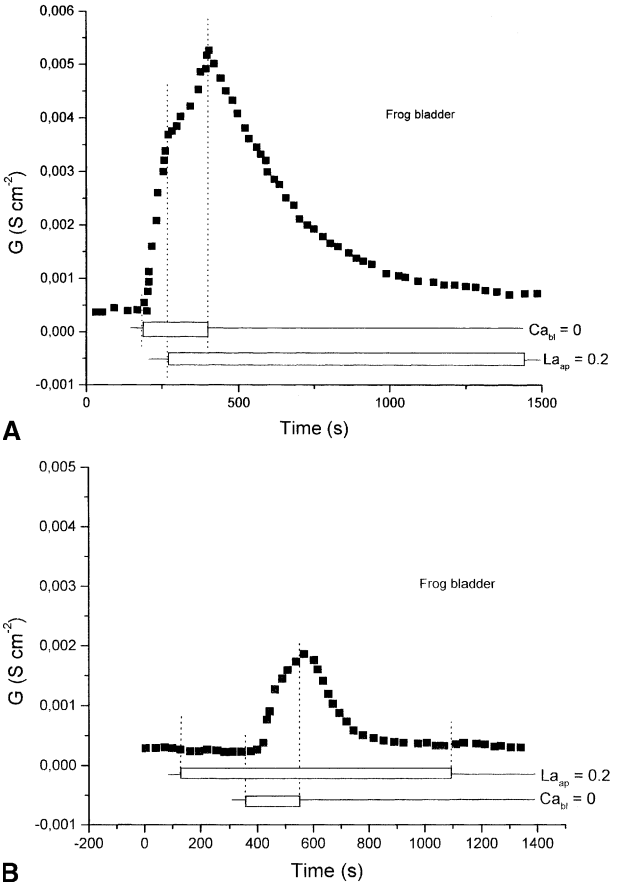

Fig. 3. (*A*) Representative experiment (of a group of 6) in frog urinary bladder on the action of La^{3+} (0.2 mM) added to the apical solution during the rising phase of *G* in an FCSA. The response is characterized by a fast increase of *G* when $Ca^{2+}{}_{bl}$ is removed; the increase slows down when $La^{3+}{}_{ap}$ is added. Finally, return of $Ca^{2+}{}_{bl}$ to normal levels leads to a complete recovery of low *G* even in the presence of $La^{3+}{}_{ap}$. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer. (*B*) Representative experiment (of a group of 6) in frog urinary bladder on the action of La^{3+} (0.2 mM) added to the apical solution before an FCSA is conducted. $La^{3+}{}_{ap}$ had no effect on the steady-state basal level of *G*. However, when an FCSA was performed it can be seen that *G* increase in response to $Ca^{2+}{}_{bl}$ removal was markedly reduced, indicating that $La^{3+}{}_{ap}$ shows an agonistic effect similar to that of apical Ca^{2+}. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

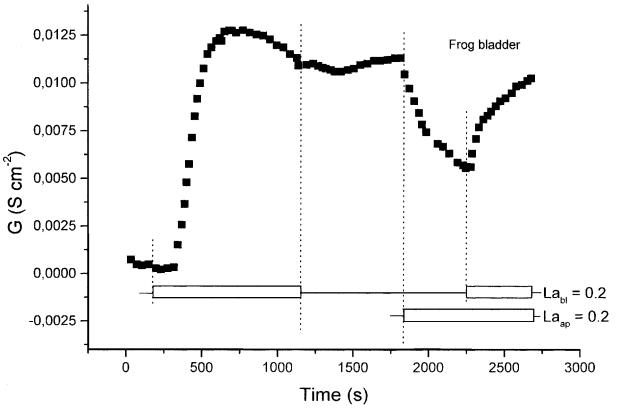

Fig. 4. Representative experiment (of a group of 6) in frog urinary bladder on the action of La^{3+} (0.2 mM) added to the basolateral solution followed by its withdrawal after about 1000 s. The response is characterized by a fast increase of *G*, which remains high after $La^{3+}{}_{bl}$ removal. A later addition of La^{3+} to the apical solution at the same concentration (0.2 mM) triggers a decline of *G*, which is then interrupted and reversed when a same concentration of La^{3+} is added to the basolateral solution. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

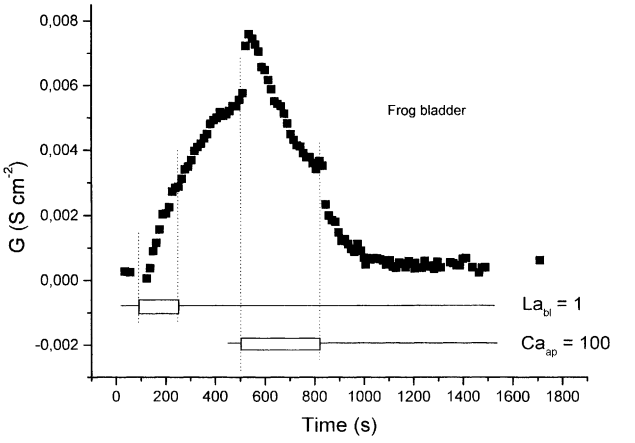

Fig. 5. Representative experiment (of a group of 4) in frog urinary bladder on the action of La^{3+} (1 mM) added to the basolateral solution, followed by its withdrawal after about 180 s. The response is characterized by an increase of *G*, which remains increasing after $La^{3+}{}_{bl}$ removal, due to the high La^{3+} concentration used. A later addition of Ca^{2+} to the apical solution (100 mM) triggers a decline of *G*, which continues to a full recovery to initial values after $Ca^{2+}{}_{ap}$ removal. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

can be seen that in the absence of $Ca^{2+}{}_{bl}$, during the phase in which *G* is increasing, La^{3+} addition to the apical bathing fluid triggers a halt of the process of junction opening and promotes a partial recovery of the TJ seal, which apparently is not much affected when $Ca^{2+}{}_{bl}$ is returned to normal levels.

Results from A6 Cell Monolayers

A representative experiment on the action of $La^{3+}{}_{bl}$ on A6 cell monolayer TJs is shown in Fig. 7, in which the first run is a normal FCSA response for the sake of control. Subsequently, after *G* had returned to basal levels, $Ca^{2+}{}_{bl}$ was replaced by $La^{3+}{}_{bl}$ at a much lower concentration (0.1 mM). In contrast to what would be expected from the results in frog urinary bladders, the presence of $La^{3+}{}_{bl}$ renders TJs insensitive to $Ca^{2+}{}_{bl}$ removal, a procedure that causes TJ opening in control tissues, both A6 cell

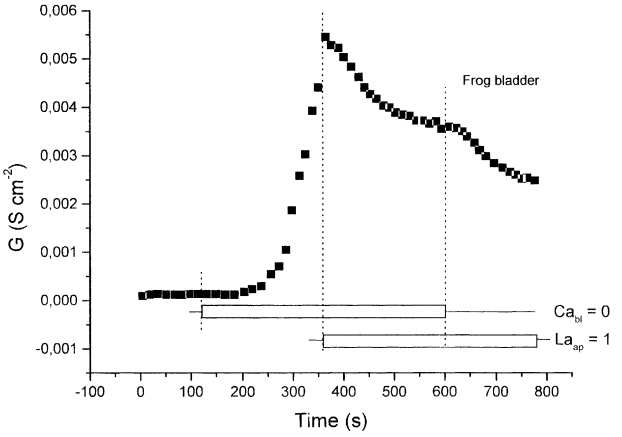

Fig. 6. Representative experiment (of a group of 6) in frog urinary bladder on the action of La^{3+} (1 mM) added to the apical solution in the course of *G* increase during an FCSA. The response is characterized by an abrupt stop of *G* increase followed by a recovery process that continues when $Ca^{2+}{}_{bl}$ is returned. The

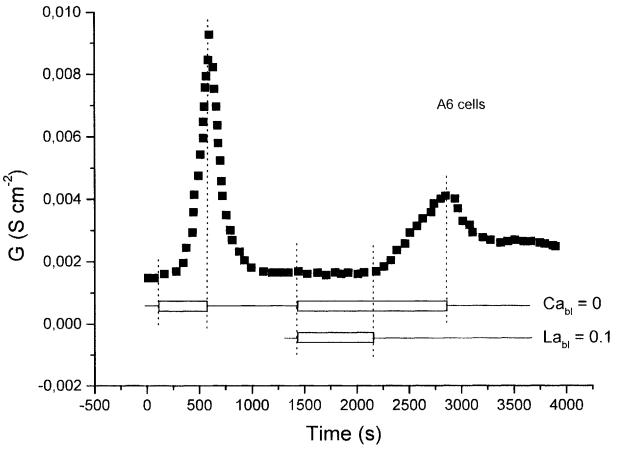

Fig. 7. Representative experiment (of a group of 6) in A6 cell monolayers on the action of La^{3+} (0.1 mM) added to the basolateral solution. The first run is a control FCSA for the sake of comparison. The second run shows that total Ca^{2+} (1 mM) substitution by a much lower La^{3+} concentration (0.1 mM) in the basolateral solution does not lead to *G* increase, indicating a high-affinity agonistic effect of La^{3+} in A6 cell monolayers. A subsequent $La^{3+}{}_{bl}$ removal triggers *G* increase at a slower rate as compared to the control FCSA. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

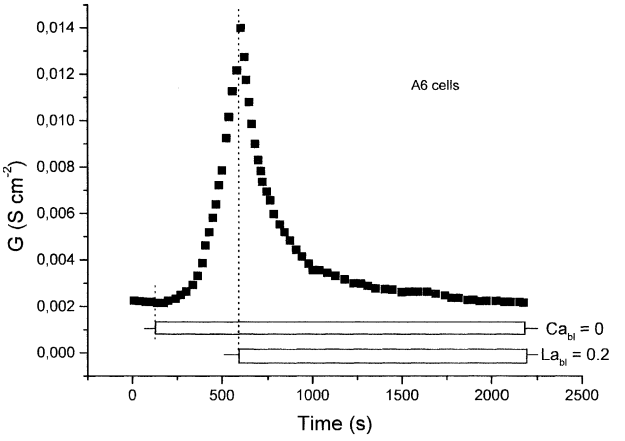

Fig. 8. Representative experiment (of a group of 6) in A6 cell monolayers on the action of La^{3+} (0.2 mM) added to the basolateral solution in the course of *G* increase during an FCSA. The response is characterized by an abrupt halt of *G* increase followed by a complete recovery of low *G* in the total absence of $Ca^{2+}{}_{bl}$. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

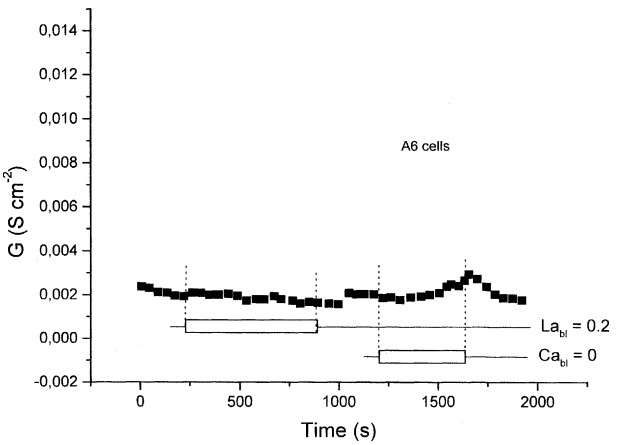

Fig. 9. Representative experiment (of a group of 5) in A6 cell monolayers on the action of La^{3+} (0.2 mM) added to the basolateral solution. It can be seen that addition of La^{3+} to the basolateral solution not only is ineffective in causing TJ opening but, in addition, after its removal a residual effect remains for a long period, indicated by the fact that a subsequent $Ca^{2+}{}_{bl}$ removal fails to induce a prompt TJ opening response.

monolayers and urinary bladders. A subsequent $La^{3+}{}_{bl}$ removal triggers TJ opening, indicating that La^{3+} in A6 cells was acting on the basolateral side as a Ca^{2+} agonist, promoting the sealing of TJs. From the time course of *G* increase after $La^{3+}{}_{bl}$ removal, it can be concluded that a residual effect of La^{3+} persists, indicated by a slower rate of *G* increase as compared to the first run. The Ca^{2+}-agonistic effect of $La^{3+}{}_{bl}$ in A6 cell monolayers can be clearly demonstrated in Fig. 8, where TJs, opened in response to $Ca^{2+}{}_{bl}$ removal, close completely in response to addition of La^{3+} (0.2 mM) to the basolateral solution. The fact that a concentration of La^{3+} (0.2 mM), one fifth of the normal $Ca^{2+}{}_{bl}$ concentration, leads to a complete sealing of TJs is an indication of the high affinity of La^{3+} for the Ca^{2+}-binding sites. This high affinity is better evidenced in the representative experiment of Fig. 9, in which addition of La^{3+} (0.2 mM) to the basolateral solution not only is ineffective in causing TJ opening but, in addition,

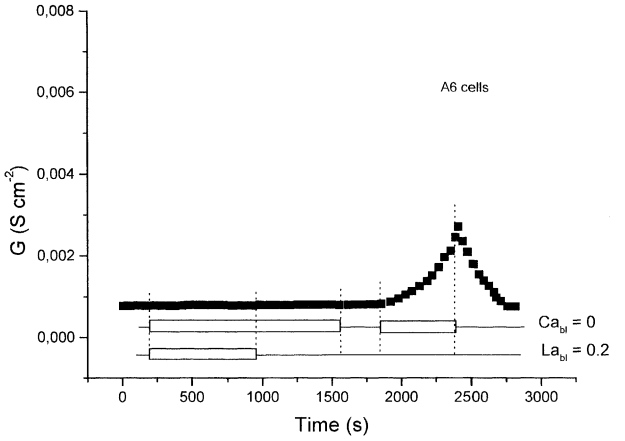

Fig. 10. Representative experiment (of a group of 5) in A6 cell monolayers on the action of La^{3+} (0.2 mM) added to the basolateral solution. It can be seen that replacement of $Ca^{2+}{}_{bl}$ (1 mM) by $La^{3+}{}_{bl}$ at a much lower concentration (0.2 mM) does not cause TJ opening, in consonance with the high-affinity Ca^{2+}-agonistic effect of La^{3+} for the binding sites already shown. In addition, a residual effect characterized by absence of junction opening when both Ca^{2+} and La^{3+} are removed can be observed. This experiment also shows that a brief exposure of the basolateral side to Ca^{2+} is sufficient to dislodge La^{3+} from the binding sites leading to TJ opening when $Ca^{2+}{}_{bl}$ is removed.

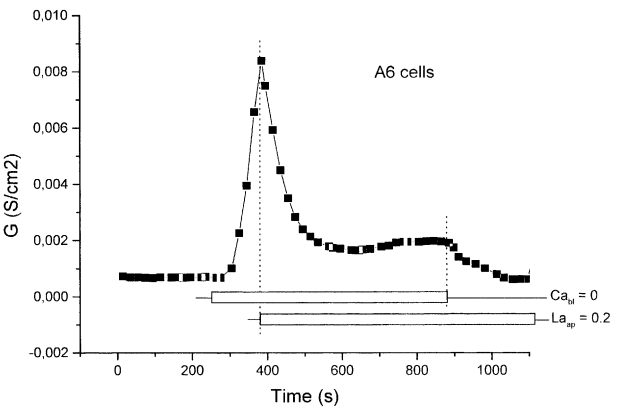

Fig. 11. Representative experiment (of a group of 5) in A6 cell monolayers on the action of La^{3+} (0.2 mM) added to the apical solution in the course of *G* increase of an FCSA. The response is characterized by a halt in the process of junction opening followed by almost complete recovery of the junction sealing. Finally, return of $Ca^{2+}{}_{bl}$ to normal levels leads to a complete recovery of *G* even in the presence of $La^{3+}{}_{ap}$. The apical solution was KCl 75 mM and the basolateral solution, NaCl HEPES Ringer.

after its removal a residual effect remains for a long period, indicated by the fact that a subsequent $Ca^{2+}{}_{bl}$ removal failed to induce a prompt TJ opening response.

In another representative experiment (Fig. 10), it can be seen that replacement of $Ca^{2+}{}_{bl}$ (1 mM)

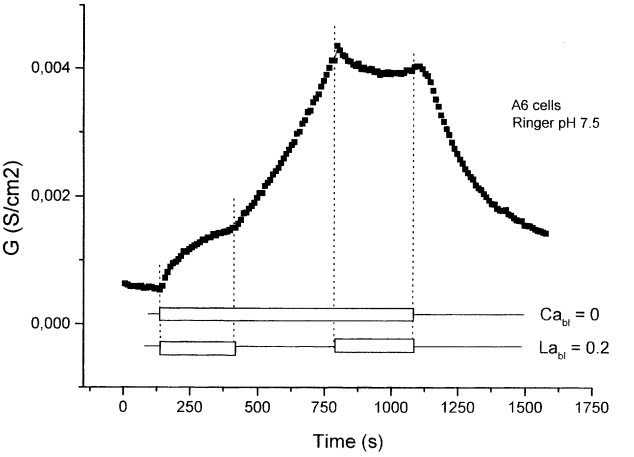

Fig. 12. Representative experiment (of a group of 6) in A6 cell monolayers on the action of La^{3+} (0.2 mM) added to the basolateral solution. Experiments were carried out to appraise possible interactions of La^{3+} and H^{+} for the binding sites that control the TJs. The pH of the Ringer's solution was lowered from the standard value of 8.2 to 7.5, causing a decrease of the La^{3+}-agonistic effect, since at this lower pH value $La^{3+}{}_{bl}$ at 0.2 mM no longer is able to maintain the TJs sealed in the absence of $Ca^{2+}{}_{bl}$ or to cause a complete junction resealing, as was the case at pH 8.2.

by $La^{3+}{}_{bl}$ at a much lower concentration (0.2 mM) does not cause TJ opening, indicating a high-affinity Ca^{2+}-agonistic effect of La^{3+} for the binding sites. In addition, a residual effect, characterized by absence of junction opening when both Ca^{2+} and La^{3+} are removed, can be observed. This experiment also shows that a brief exposure of the basolateral side to Ca^{2+} is sufficient to dislodge residual La^{3+} ions from the binding sites, leading to TJ opening when $Ca^{2+}{}_{bl}$ is removed. These results indicate that La^{3+} strongly binds to the Ca^{2+} sites and that La^{3+} and Ca^{2+} display a competitive interaction for the binding sites.

In order to test the effect of $La^{3+}{}_{ap}$ in A6 cell monolayers, experiments were performed in which the TJs were opened by $Ca^{2+}{}_{bl}$ removal and subsequently La^{3+} was added to the apical bathing fluid. In 5 experiments, $La^{3+}{}_{ap}$ (0.2 mM) added during the phase of TJ opening in an FCSA halted the process of junction opening, inducing almost complete recovery of the TJ seal. A representative experiment is shown (Fig. 11).

Experiments were carried out to check possible interactions of La^{3+} and H^{+} for the binding sites that control the TJs. Lowering the pH of the Ringer's solution from the standard value of 8.2 to 7.5 caused a decrease of the La^{3+} agonistic effect, as shown in Fig. 12, where $La^{3+}{}_{bl}$ at 0.2 mM no longer is able to maintain the TJs sealed in the absence of $Ca^{2+}{}_{bl}$ or to cause a complete junction resealing, as was the case at pH 8.2 (Fig. 8).

Discussion

The present results address the interactions of La^{3+} with the Ca^{2+}-binding sites that control the early events of TJ opening and closing, in order to probe for differences in La^{3+} binding selectivity. Interactions of La^{3+} with living tissues have been studied since a long time in different preparations (Weiss, 1974). The present work is a comparative study in frog urinary bladders and mature A6 cell monolayers. The results were obtained by the "fast Ca^{2+} switch assay" (FCSA) (*see* Methods) (Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997; Lacaz-Vieira et al., 1999; Lacaz-Vieira, 2000; Kassab Jr., et al., 2002), which allows assessment of the early kinetic events of TJ dynamics as well as evaluation of the effects of drugs and procedures affecting the control of TJ dynamics, whereas it prevents more complex regulatory responses that are involved in long-term experiments.

A previous study provided compelling evidence that metal ions interact with the Ca^{2+}-binding sites that control the TJs, with different effects (Lacaz-Vieira, 1997). In that study it was shown that La^{3+} addition to the basolateral bathing solution ($La^{3+}{}_{bl}$) of frog urinary bladders, in the presence of a normal Ca^{2+} concentration, led to opening of TJs, a process that reverses when $La^{3+}{}_{bl}$ was removed. This is a relevant finding bearing in mind that La^{3+} is frequently used as a paracellular tracer or as a paracellular electron dense marker in the study of epithelial and endothelial membranes (*see* Introduction). The fact that La^{3+} by itself can alter TJ permeability, even in the presence of a normal extracellular Ca^{2+} concentration, can lead to misleading interpretations when La^{3+} is used to probe the tightness of the paracellular seal.

In the present paper we extended the study by comparing the effects of La^{3+} on two different tissues, frog urinary bladders and A6 cell monolayers, showing the existence of a sharp difference in response. In frog urinary bladders (Fig. 1) $La^{3+}{}_{bl}$ always shows a Ca^{2+}-antagonistic effect characterized by increasing tissue electrical conductance (G), which is a fair parameter of paracellular permeability (Lacaz-Vieira & Kachar, 1996), attesting to the opening of the TJ seal. This Ca^{2+}-antagonistic effect starts to be noticed around 0.02 mM, being clearly evident above 0.06 mM. The fact that the onset of the $La^{3+}{}_{bl}$ effect, causing a reversible opening of the TJ seal, starts at concentrations much lower than the normal Ca^{2+} concentration of the Ringer's solution, is a clear evidence of a high affinity of La^{3+} for the Ca^{2+}-binding sites. In contrast to what was observed in frog urinary bladders, in A6 cell monolayers, $La^{3+}{}_{bl}$ has a clear Ca^{2+}-agonistic effect, inducing TJ sealing when $Ca^{2+}{}_{bl}$ had been previously removed (Fig. 8) or preventing TJ opening when $Ca^{2+}{}_{bl}$ is replaced by La^{3+} (Fig. 7). In both tissues the effect of $La^{3+}{}_{bl}$ is seen at a much lower concentration compared to the normal extracellular Ca^{2+} concentration, stressing its high affinity for the Ca^{2+}-binding sites, which can be understood considering the high valence of La^{3+} compared to Ca^{2+} and the very close ionic radii of both ions (Snyder et al., 1990). Multiple factors (number, type, and geometry of ligands, electrostatic interactions, cavity size and deformability of the site, dehydration of the metal and ligand) are among the variables that must be considered when metal ion selectivity of protein Ca^{2+} sites is analyzed (Snyder et al., 1990).

The kinetics of TJ opening in frog urinary bladders induced by La^{3+} differ from that induced by removing $Ca^{2+}{}_{bl}$ in an FCSA. When TJs are opening in response to $Ca^{2+}{}_{bl}$ removal, $Ca^{2+}{}_{bl}$ reintroduction leads to an abrupt halt of G increase immediately followed by the recovery process; in the La^{3+} experiments, low-G recovery triggered by La^{3+} withdrawal is always preceded by a delay that gets longer with increasing La^{3+} concentration and/or La^{3+} exposure time (Figs. 2 and 4). This delay may reflect not only the high affinity of La^{3+} for the Ca^{2+}-binding sites, but also the time course of La^{3+} wash-out from the paracellular space following its withdrawal from the basolateral solution. The fact that a short pulse of high Ca^{2+} concentration in the apical solution accelerates the recovery process that takes place after $La^{3+}{}_{bl}$ withdrawal, strongly favors the interpretation of $Ca^{2+}{}_{ap}$ entering the open TJs, dislodging La^{3+} ions from the binding sites, and prompting a rapid recovery process thereafter (Fig. 5).

Where is $La^{3+}{}_{bl}$ acting to promote its Ca^{2+}-agonistic effect in A6 cell monolayers or its antagonistic effect in frog urinary bladders? The E-cadherin, a 120 kDa transmembrane glycoprotein, belonging to the family of classical cadherins (Ranscht, 1994) which exhibit Ca^{2+}-dependent homophilic interactions (Takeichi, 1990) and five extracellular domains, each with internal sequence homology and conserved Ca^{2+}-binding motifs (Kemler, 1992) is a natural candidate. Therefore, both the Ca^{2+}-antagonistic effect of La^{3+} in frog urinary bladders or the agonistic effect in A6 cell monolayers might, in principle, result from the interaction of La^{3+} with the Ca^{2+}-binding motifs of E-cadherin. Subtle differences, yet to be identified in these sites, might be responsible for the resulting agonistic or antagonistic effect of $La^{3+}{}_{bl}$.

In frog urinary bladders, differently from its basolateral effect, apical La^{3+} exhibits a Ca^{2+}-agonistic effect (Figs. 3, 4 and 6). A similar agonist effect is also observed in A6 cell monolayers (Fig. 11). This might prompt us to postulate a dual effect of La^{3+}, depending on which side of the bladder it is acting. The effect of La^{3+} in the basolateral solution, both in frog urinary bladders and in A6 cell monolayers, is consistently coherent with the interpretation of it

acting, antagonistically or agonistically, on the Ca^{2+} binding sites of *zonula adhaerens*. In contrast, despite the fact that the effect of $La^{3+}{}_{bl}$ is clear, its site of action is still to be identified.

In A6 cell monolayers, the $La^{3+}{}_{bl}$ effect is always agonistic. Thus, in the presence of $La^{3+}{}_{bl}$ the removal of $Ca^{2+}{}_{bl}$ does not induce TJ opening as it normally does in control condition, and the subsequent $La^{3+}{}_{bl}$ removal triggers the opening of the junction seal; when G is increasing in an FCSA induced by $Ca^{2+}{}_{bl}$ removal, addition of $La^{3+}{}_{bl}$ triggers junction closure. The fact that a concentration of La^{3+} one fifth of the normal $Ca^{2+}{}_{bl}$ leads to a complete recovery of the TJ seal (Fig. 8) indicates a high affinity of La^{3+} for the Ca^{2+}-binding sites. The apparent competitive interaction between Ca^{2+} and La^{3+} might be taken as plausible evidence that both ions produce their effects on the TJs by acting on the same binding sites.

Protonation of the Ca^{2+}-binding sites by lowering the pH of the basolateral solution from the normal value of 8.2 to 7.5, causing a decrease of the agonistic effect on A6 cell monolayers, is an indication of a negatively charged binding site.

The grounds for inferring the effect of La^{3+} as a Ca^{2+} agonist in A6 cell monolayers or as a Ca^{2+} antagonist in frog urinary bladders is a complex matter. In these two structures it is clear, however, that La^{3+} interacts with the Ca^{2+}-binding sites, otherwise we would not expect the effects being observed. In A6 cells $La^{3+}{}_{bl}$ apparently replaces $Ca^{2+}{}_{bl}$ in a way that it mimics well the interaction of Ca^{2+}, triggering the cascade of events that ends in the closure of TJs. In frog bladders, La^{3+} interacts with the binding sites, dislodges Ca^{2+} with a high affinity, but this interaction is inadequate to initiate or sustain the process of junction closing. Most probably, the discrepancy in response to $La^{3+}{}_{bl}$ between the two preparations resides on subtle differences of the outer segment of E-cadherin molecules. Ca^{2+} modulates the conformation of E-cadherin, stabilizes it in its adhesive state ((Ringwald et al., 1987; Zheng et al., 2004) and mediates homophilic binding of E-cadherins (Gottardi et al., 2001). This interaction with $Ca^{2+}{}_{bl}$ is transduced across the cell membrane by a cascade of signaling reactions (Balda et al., 1991, 1993). Most probably the agonistic effect of La^{3+} in A6 cell monolayers triggers these same signaling pathways.

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