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Pulses of Cell Ca^{2+} and the Dynamics of Tight Junction Opening and Closing

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Abstract. A mathematical modeling of tight junction (TJ) dynamics was elaborated in a previous study (Kassab, F., Marques, R.P., Lacaz-Vieira, F. 2002. Modeling tight junction dynamics and oscillations. J. Gen. Physiol. 120:237–247) to better understand the dynamics of TJ opening and closing, as well as oscillations of TJ permeability that are observed in response to changes of extracellular Ca^{2+} levels. In this model, TJs were assumed to be specifically controlled by the Ca^{2+} concentration levels at the extracellular Ca^{2+} binding sites of *zonula adhaerens*. Despite the fact that the model predicts all aspects of TJ dynamics, we cannot rule out the likelihood that changes of intracellular Ca^{2+} concentration ($Ca^{2+}{}_{cell}$), which might result from changes of extracellular Ca^{2+} concentration ($Ca^{2+}{}_{ext}$), contribute to the observed results. In order to address this aspect of TJ regulation, fast Ca^{2+}-switch experiments were performed in which changes of $Ca^{2+}{}_{cell}$ were induced using the Ca^{2+} ionophore A23187 or thapsigargin, a specific inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase. The results indicate that the ionophore or thapsigargin per se do not affect basal tissue electrical conductance (G), showing that the sealing of TJs is not affected by a rise in $Ca^{2+}{}_{cell}$. When TJs were kept in a dynamic state, as partially open structures or in oscillation, conditions in which the junctions are very sensitive to disturbances that affect their regulation, a rise of $Ca^{2+}{}_{cell}$ never led to a decline of G, indicating that a rise of $Ca^{2+}{}_{cell}$ does not trigger per se TJ closure. On the contrary, always the first response to a rise of $Ca^{2+}{}_{cell}$ is an increase of G that, in most cases, is a transient response. Despite these observations we cannot assure that a rise of $Ca^{2+}{}_{cell}$ is without effect on the TJs, since an increase of $Ca^{2+}{}_{cell}$ not only causes a transient increase of G but, in addition, during oscillations a rise of $Ca^{2+}{}_{cell}$ induced by the Ca^{2+} ionophore transiently halted the oscillatory pattern of TJs. The main conclusion of this study is that TJ closure that is observed when basolateral Ca^{2+} concentration ($Ca^{2+}{}_{bl}$) is increased after TJs were opened by $Ca^{2+}{}_{bl}$ removal cannot be ascribed to a rise of $Ca^{2+}{}_{cell}$ and might be a consequence of Ca^{2+} binding to extracellular Ca^{2+} sites.

Key words: Tight junction — Calcium — Ca^{2+} — Oscillations — Cell calcium — Ca^{2+} ionophore — Thapsigargin

Introduction

Despite the central role of intercellular junctions in epithelial function, much remains to be known about the signalling processes involved in junction regulation. The assembly of intercellular junctions has been studied in different preparations and it has been proposed that their formation is initiated by cadherin-mediated cell-cell contact and the formation of the *zonula adhaerens* followed by the assembly of desmosomes and tight junctions (TJs). Ca^{2+} is essential for cells to develop and maintain these junctions. When extracellular Ca^{2+} is removed the cell-cell connections generally become loose and for long exposure to Ca^{2+}-free medium multicellular organization is destroyed. Several studies have emphasized the role of extracellular Ca^{2+} on the stability of mature tight junctions in natural epithelia (Sedar & Forte, 1964; Hays, Singer & Malamed, 1965; Galli, Camilli, Meldolesi, 1976; Meldolesi et al., 1978; Palant et al., 1983; Pitelka, Taggert & Hamamoto, 1983; Lacaz-Vieira, 1997) and on the development of new TJs in cell cultures in confluence (Cereijido et al., 1980;

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Martinez-Palomo et al., 1980; Cereijido, Meza & Martinez-Palomo, 1981; González-Mariscal, Chávez de Ramirez & Cereijido, 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+} on the dynamics of TJs, major questions are still pending relative to the precise steps in the signalling chain, mediating specific assembly stages. The relative importance of extracellular Ca^{2+} (González-Mariscal et al., 1990; Contreras et al., 1992b; Gorodeski et al., 1997) versus intracellular Ca^{2+} concentration changes (Bhat et al., 1993; Stuart et al., 1994; Jovov et al., 1994; Stuart et al., 1996) on the dynamics of TJ opening and closing is not yet clearly characterized. The cell adhesion molecule E-cadherin, which is particularly concentrated at the *zonula adhaerens* (Bollers, Vestweber & Kemler, 1985), apparently plays a key role as an extracellular Ca^{2+}-binding molecule that modulates the formation and maintenance of the epithelial junctional complex (Gumbiner et al., 1988; Troxell et al., 1999). Ca^{2+} influences the conformation of E-cadherin and stabilizes it in its adhesive state (Ringwald et al., 1987) by interacting with a negatively charged pocket formed by three sequences of highly conserved residues well positioned to ligate Ca^{2+} (Overduin et al., 1995).

Ca^{2+} interaction with E-cadherin molecules is transduced inside the cells by a cascade of signalling reactions involving different components (Balda et al., 1991, 1993). The hierarchical regulation of junctional-complex assembly is, however, not absolute, as inhibition of cadherin function has both positive and negative effects on tight junction assembly (Troxell et al., 2000).

In a previous study in the frog urinary bladder (Lacaz-Vieira & Kachar, 1996) it was shown that apical Ca^{2+} may activate the TJ sealing mechanism, an effect that is not impaired by the apical presence of Ca^{2+}-channel blockers (nifedipine, verapamil, Mn^{2+} or Cd^{2+}), suggesting that junction resealing due to $Ca^{2+}{}_{ap}$ in the frog urinary bladder is not mediated by Ca^{2+} entering the cells through the apical membrane. Most likely the response to $Ca^{2+}{}_{ap}$ resulted from Ca^{2+} entering partially disrupted TJs, reaching the *zonula adhaerens* Ca^{2+} receptors (E-cadherins) and triggering TJ resealing. It has been shown that protein kinase C (PKC), particularly PKC zeta, which may be part of *zonula occludens* (Dodane & Kachar, 1996), plays a significant role in the control of the dynamics of TJ assembly and disassembly (Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 2000; Lacaz-Vieira & Jaeger, 2001). To better understand the dynamics of TJ opening and closing in response to changes of the extracellular Ca^{2+} levels and the oscillations of TJ permeability (Lacaz-Vieira, 2000) we have elaborated a mathematical modeling of TJ dynamics to account for the responses to changes of $Ca^{2+}{}_{bl}$ and $Ca^{2+}{}_{ap}$ (Kassab, Jr., Marques & Lacaz-Vieira, 2002). In this model TJs were assumed to be specifically controlled by the Ca^{2+}-concentration level at the Ca^{2+}-binding sites of *zonula adhaerens* (Gumbiner et al., 1988). In addition, the model predicted different aspects of TJ dynamics in response to changes of extracellular Ca^{2+} concentration; in particular, this model supports our previous hypothesis that oscillations of G, which occur in response to a step rise of apical Ca^{2+} concentration in a fast Ca^{2+}-switch assay, resulted from oscillatory opening and closing of the TJ barrier (Lacaz-Vieira, 2000).

The fact that in our experience, both in frog urinary bladder and in A6 cell monolayers, changes of extracellular Ca^{2+} levels dramatically affect the dynamical behavior of TJs does not allow us to rule out the possibility that changes of intracellular Ca^{2+} concentration, which might result from changes of extracellular Ca^{2+} levels, also contribute to the observed results. In order to address this aspect of TJ regulation, fast Ca^{2+}-switch experiments were performed in which changes of $Ca^{2+}{}_{cell}$ were induced using the Ca^{2+} ionophore A23187 and thapsigargin, a specific inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase.

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% foetal bovine serum (Sigma, 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 opposite side of the support filters 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 chamber (Castro, Sesso & Lacaz-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 inner bathing solution was NaCl Ringer's solution with the following composition (in mM): NaCl 115, $KHCO_3$ 2.5, and $CaCl_2$ 1.0. The apical bathing fluids were simple salt solutions, non-buffered, prepared with glass-distilled water, having a 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). The apical solution was KCl 75 mM in order to eliminate Na^+ from this solution, ruling 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.

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). A23187: Ca^{2+} ionophore was dissolved in DMSO and added to the apical solution at 1 μM final concentration. Thapsigargin: was dissolved in DMSO and added to the basal solution at 1 μM final concentration.

Statistics

The results are presented as mean ± standard error of the mean (SEM). 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. We calculated G using a data analysis and technical graphing software Origin™ (version 5) (Microcal Software, Inc.). The clamping current was initially smoothed by adjacent 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 study aimed to test the effects of changes in $Ca^{2+}{}_{cell}$ on the dynamics of TJ opening and closing in response to a fast Ca^{2+}-switch assay (FCSA) (Lacaz-Vieira, 1997, 2000; Lacaz-Vieira et al., 1999; Kassab, Jr. et al., 2002). The use of two different preparations (A6 cell monolayers and frog urinary bladders) resulted from the fact that they differ in some aspects regarding the response to a FCSA. Thus, oscillations of TJ permeability are clearly observed in frog urinary bladders (Lacaz-Vieira, 2000; Kassab, Jr. et al., 2002) but were never seen in cultured A6 cell monolayers. Besides, the effects of PKC inhibitors differ when these preparations are compared (Lacaz-Vieira, 2000; Lacaz-Vieira & Jaeger, 2001).

The FCSA was used as a routine procedure to induce TJ opening and closing. Pulses of $Ca^{2+}{}_{cell}$, delivered at different times during tissue response to the FCSA (including in the control condition prior to the FCSA), were used to evaluate how changes in $Ca^{2+}{}_{cell}$ might affect TJ dynamical behavior.

$Ca^{2+}{}_{cell}$ was raised by using two different procedures: (i) allowing Ca^{2+} to enter the cells through the apical membrane using the Ca^{2+} ionophore

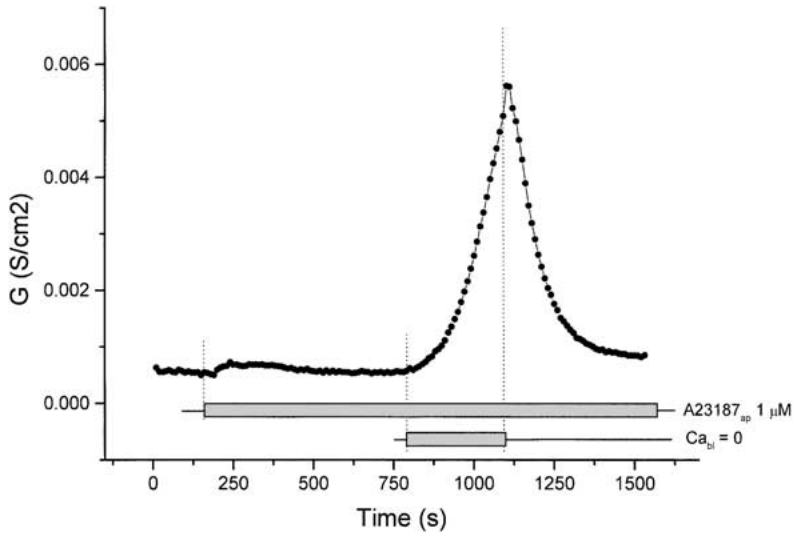


Fig. 1. Representative experiment (of a group of 6) in A6 cell monolayers on the effect of the Ca^{2+} ionophore (A23187) added to the apical bathing fluid (1 μM) on basal tissue electrical conductance (*G*) and the dynamics of TJ opening and closing in an FCSA . The apical solution was KCl 75 mM, and the basolateral, NaCl-Ringer's solution. The rectangles indicate the period in which the concentrations are those specified in the figure at the right. When units are not specified, the concentrations are in mM.

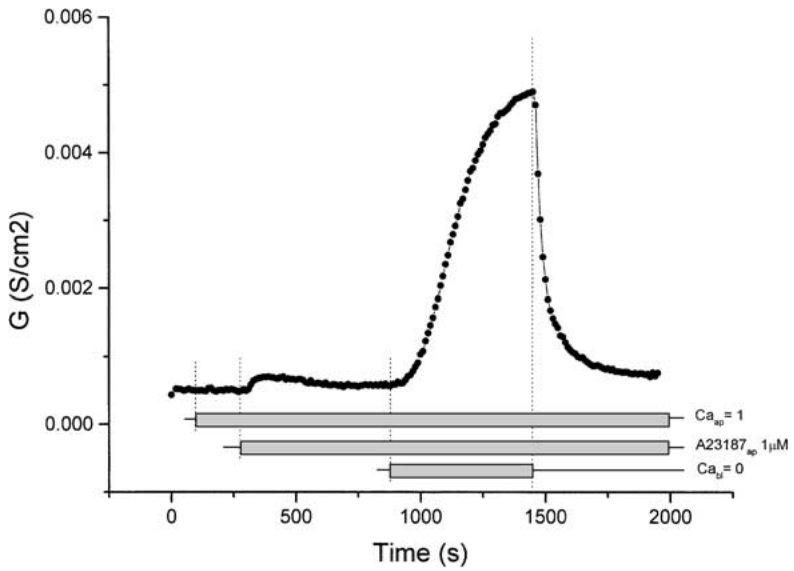


Fig. 2. Representative experiment (of a group of 5) on the effect of the Ca^{2+} ionophore (A23187) added to the apical bathing fluid on the dynamics of TJ opening and closing in an FCSA in A6 cell monolayers. The apical solution was KCl 75 mM, and the basolateral, NaCl-Ringer's solution. The rectangles indicate the period in which there were changes in concentration, which are then specified at the right. When units are not specified, the concentrations are in mM.

A23187 (Reed & Lardy, 1972) or (ii) releasing Ca^{2+} stores by blocking the sarco-endoplasmic reticulum Ca^{2+}-ATPase with the specific inhibitor thapsigargin (Stuart et al., 1996; Jan et al., 1999).

TJs in the Closed State

Experiments with Ca^{2+} Ionophore A23187

These experiments were undertaken to evaluate the effects of increasing $Ca^{2+}{}_{cell}$ on the steady-state tissue electrical conductance (*G*) with the TJs in a closed state. The Ca^{2+} ionophore (A23187) was added to the apical solution at 1 μM concentration, in the absence or the presence of $Ca^{2+}{}_{ap}$, in both preparations, A6 cell monolayers and frog urinary bladders. Representative experiments in A6 cell monolayers are shown in Fig. 1 and Fig. 2 where the ionophore was tested in the absence and in the presence of 1 mM $Ca^{2+}{}_{ap}$, respectively. Similar results were observed in frog urinary bladders (*data not shown*). The slower time course of TJ opening in the presence of $Ca^{2+}{}_{ap}$ (Fig. 2) when compared to the condition in the absence of $Ca^{2+}{}_{ap}$ (Fig. 1) results exclusively from the presence of $Ca^{2+}{}_{ap}$, as shown previously (Lacaz-Vieira, 1997). From these experiments we may conclude that *G* was weakly affected by the ionophore in all cases, Indicating that the TJs remain in their closed state and that the Ca^{2+} ionophore per se does not increase significantly the conductance of the apical membrane, both in absence or presence of $Ca^{2+}{}_{ap}$. In addition, the ionophore did not affect the kinetics of TJ opening and closing in response to a FCSA as compared to a condition in its absence. Mean values are presented in Fig. 3 columns *A* and *B*.

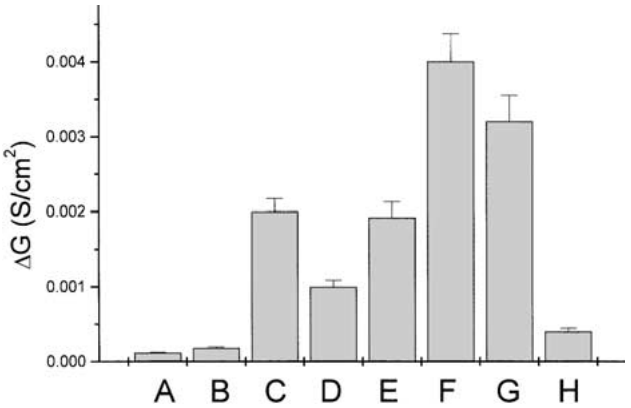


Fig. 3. Mean values of ΔG (difference between G peak values after drug addition (A23187 or thapsigargin) and G values 5 s prior to drug addition to the proper bathing solution). (*A*) A6 cell monolayers. A23187 added during the initial control steady-state to the apical solution containing no Ca^{2+}; group relative to Fig. 1 ($n = 6$). (*B*) A6 cell monolayers. A23187 added during the initial control steady-state to the apical solution containing Ca^{2+}; group relative to Fig. 2 ($n = 5$). (*C*) A6 cell monolayers, A23187 added during the metastable condition to the apical solution containing Ca^{2+}; group relative to Fig. 4*B* ($n = 5$). (*D*) A6 cell monolayers, A23187 added during the metastable condition to the apical solution containing Ca^{2+} group relative to Fig. 4*C* ($n = 6$). (*E*) Frog urinary bladders. A23187 added during the metastable condition to the apical solution containing Ca^{2+}; group relative to Fig. 4*D* ($n = 6$). (*F*) Frog urinary bladders. Thapsigargin added during the metastable condition to the apical solution containing Ca^{2+}; group relative to Fig. 5 ($n = 8$). (*G*) A6 cell monolayers. Thapsigargin added during the metastable condition to the apical solution containing Ca^{2+}; group relative to Fig. 6 ($n = 4$). (*H*) A6 cell monolayers. Thapsigargin added during the metastable condition to the apical solution containing Ca^{2+}; group relative to Fig. 7 ($n = 5$). Cells were pretreated with BAPTA-AM

Experiments with Thapsigargin

In these experiments a rise of $Ca^{2+}{}_{cell}$ was induced by pretreating frog urinary bladders (group of 5) with thapsigargin at 1 μM concentration in the basal solution. No effect was observed on the steady-state tissue electrical conductance. An FCSA induced 15 min after thapsigargin treatment developed as in control untreated bladders. These experiments allow the conclusion that a rise of $Ca^{2+}{}_{cell}$ induced by blocking the sarco-endoplasmic Ca^{2+}-ATPase does not affect the steady-state G nor the pattern of TJ opening in response to $Ca^{2+}{}_{bl}$ removal and closing when $Ca^{2+}{}_{bl}$ is reintroduced.

TJs in Partially Open Metastable Condition

Experiments with Ca^{2+} Ionophore A23187

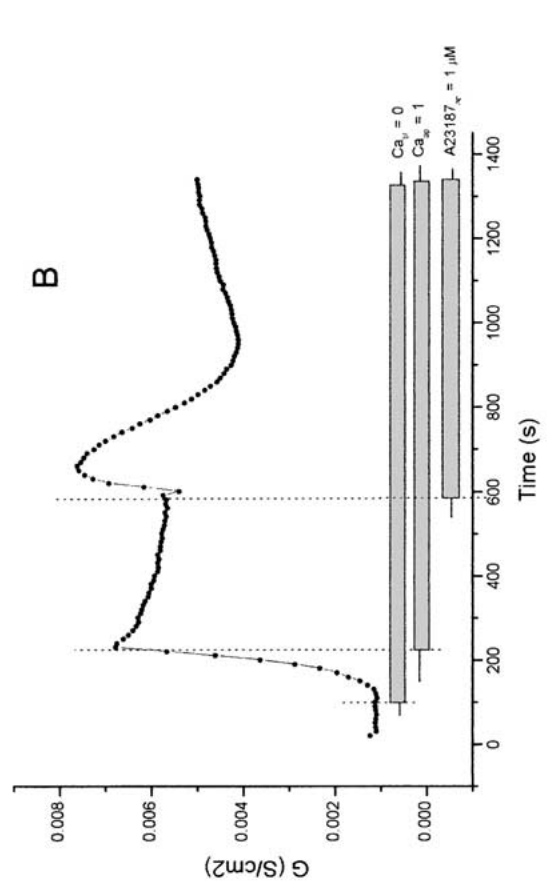
These experiments were carried out to analyze the effects of a sudden rise of $Ca^{2+}{}_{cell}$ on partially opened TJs maintained in a metastable condition that was obtained through the opening of TJs by removal of $Ca^{2+}{}_{bl}$ and then halting the process by addition of $Ca^{2+}{}_{ap}$ (Lacaz-Vieira & Jaeger, 2001). This is a nice condition to test procedures that might affect the dynamics of TJs since TJ permeability might drift to higher or lower values, or remain unchanged, depending on tissue response to the test procedure.

Experiments were performed in A6 cell monolayers. The rise of $Ca^{2+}{}_{ap}$, depending on its concentration, halts the TJ opening process in an FCSA with G stabilizing at a value higher than that prior to $Ca^{2+}{}_{bl}$ removal (Fig. 4*A*). We used this particular condition to test the effects of a sudden rise of $Ca^{2+}{}_{cell}$ on the evolution of tissue electrical conductance. The ionophore was added to the apical solution when G had attained a new stable condition. It can be observed for a representative experiment that a rise of cell Ca^{2+} induced by the ionophore in this particular condition (Fig. 4*B*) caused a conspicuous transient biphasic response characterized by an initial rise of G followed by a transient decline and a latter stabilization near its values prior to ionophore application. A similar behavior was also observed in frog urinary bladders. When TJ opening is induced in the presence of $Ca^{2+}{}_{ap}$ the opening process is slower than that seen in its absence, but a metastable state is also achieved in which G is larger than that on the basal initial condition (Lacaz-Vieira, 2000). If Ca^{2+} ionophore is added to the apical solution it also elicits a conspicuous transient response similar to that described above. Figs. 4*C* and 4*D* are representative experiments carried out in A6 cell monolayers and frog urinary bladders, respectively. Mean values are presented in Fig. 3 columns *C, D* and *E*.

All these results indicate that in the metastable condition, where TJs can swing to more closed or open states, a rise of cell Ca^{2+} elicits a conspicuous transient junctional biphasic response without, however, causing TJ closure. Thereupon, we can conclude that in both, A6 cell monolayers and frog urinary bladders, the closure of TJs that takes place in response to addition of Ca^{2+} to the basolateral solution cannot be ascribed as directly resulting from a rise of $Ca^{2+}{}_{cell}$ concentration that could have resulted from Ca^{2+} entering through a permeable basolateral membrane. Consequently, TJ closure might result from an extracellular action of Ca^{2+} acting on extracellular Ca^{2+} binding sites of E-cadherin molecules of *zonula adhaerens* (Boiler et al., 1985; Gumbinere et al., 1988), as previously proposed (Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997; Kassab, Jr. et al., 2002)

Experiments with Thapsigargin

These experiments were carried out to test a different way of increasing $Ca^{2+}{}_{cell}$, other than using Ca^{2+} ionophores, and follow its effect on TJs that were


A
G (S/cm2)
Time (s)
Ca_ic = 0
Ca_ec = 1

B
G (S/cm2)
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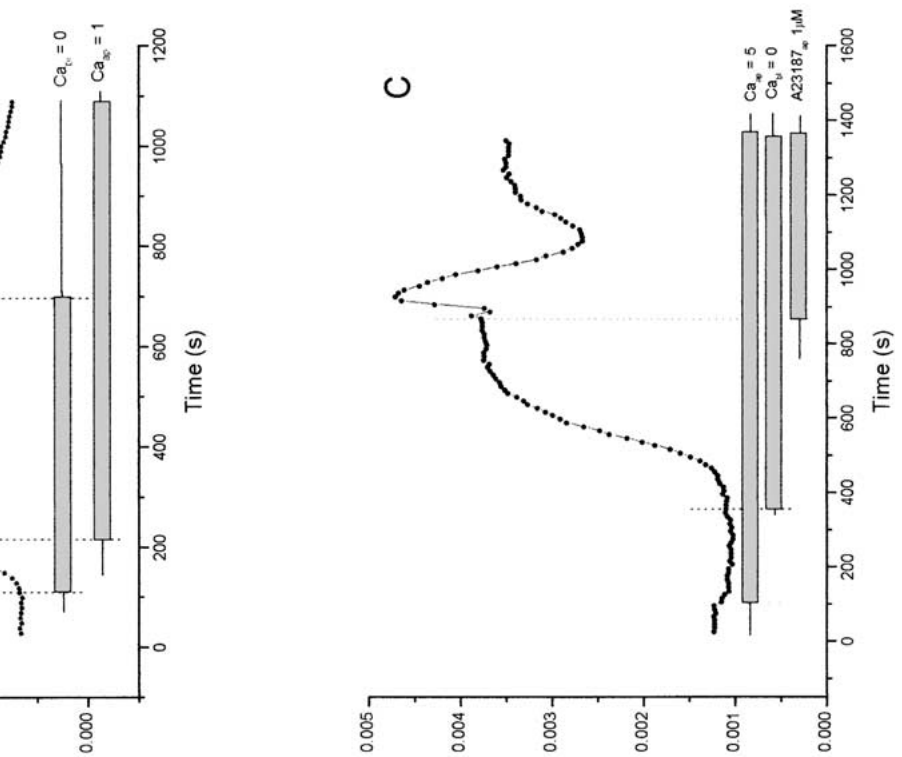
Ca_ec = 1
A23187_ec = 1 µM


C
G (S/cm2)
Time (s)
Ca_ec = 5
Ca_ic = 0
A23187_ec 1µM

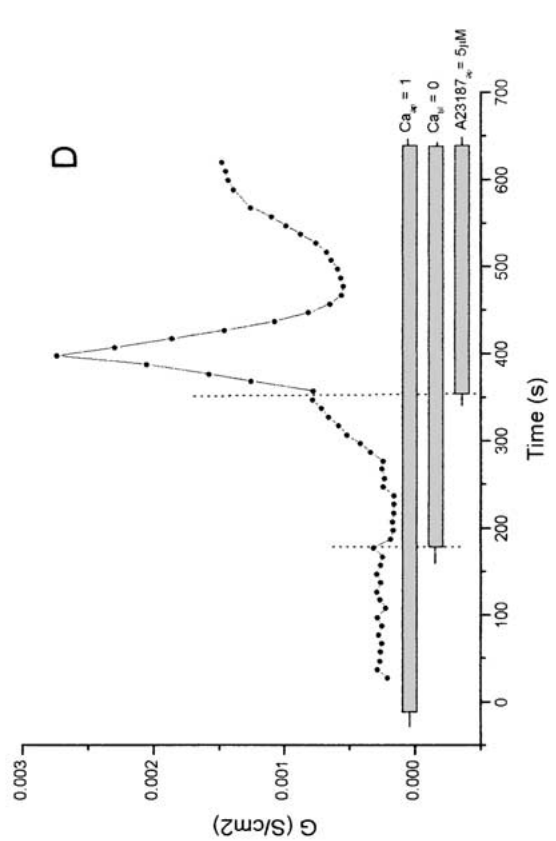

D
G (S/cm2)
Time (s)
Ca_ec = 1
Ca_ic = 0
A23187_ec = 5µM

Fig. 4. Representative experiments on the effect of a pulse of cell Ca^{2+}, with the TJs held in steady-state partially opened condition. (*A*) Control experiment in A6 cell monolayers (of a group of 10) showing the effect of rising $Ca^{2+}{}_{ap}$ concentration (1 mM) on the process of TJ opening in response to $Ca^{2+}{}_{bl}$ removal, showing a halt in the TJ opening process and stabilization of *G* in a new steady state. (*B*) Similar to the experiment (*A*), in A6 cell monolayers (of a group of 5), except that the Ca^{2+} ionophore A23187 (1 μM) was added to the apical solution when the new steady state after $Ca^{2+}{}_{ap}$ addition was attained. (*C*) In this example, in A6 cell monolayers (of a group of 6), the new steady state was reached by removing $Ca^{2+}{}_{bl}$ with Ca^{2+} already present in the apical solution (5 mM). Afterwards, the ionophore A23187 (1 μM) was added to the apical solution. (*D*) Similar to experiment (*C*), except that it was performed in a frog urinary bladder and $Ca^{2+}{}_{ap}$ was 1 mM (of a group of 6).

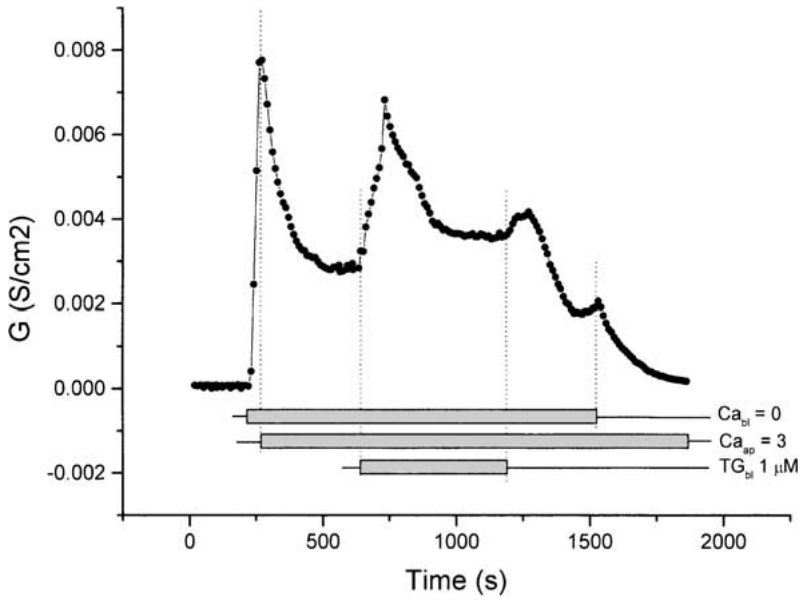


Fig. 5. Representative experiment (of a group of 8) performed in frog urinary bladders on the effect of thapsigargin (TG) added to the basolateral solution (1 μM) when TJs were kept in the metastable condition, as already described.

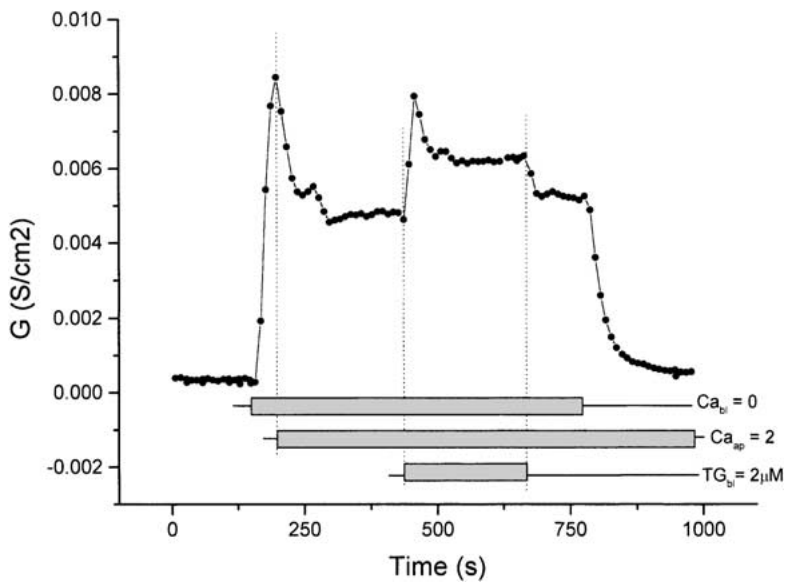


Fig. 6. Representative experiment (of a group of 4) performed in A6 cell monolayers on the effect of thapsigargin (TG) added to the basolateral solution (2 μM) when TJs were kept in the metastable condition, as already described.

maintained partially open in a metastable condition, as described above. Once a new *G* steady state was reached, thapsigargin (TG), a specific inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase, was added to the inner bathing solution (1 μM), allowing an increase of $Ca^{2+}{}_{cell}$ (Jan et al., 1999). In frog urinary bladders, a rapid transient increase of *G* occurred upon addition of TG followed by a recline to values near those observed prior to the addition of the inhibitor, as shown for a representative experiment in A6 cells (Fig. 5). Occasionally, a sustained increase of *G* was observed, as shown for a representative experiment in A6 cell monolayers (Fig. 6). TG withdrawal was normally accompanied by a

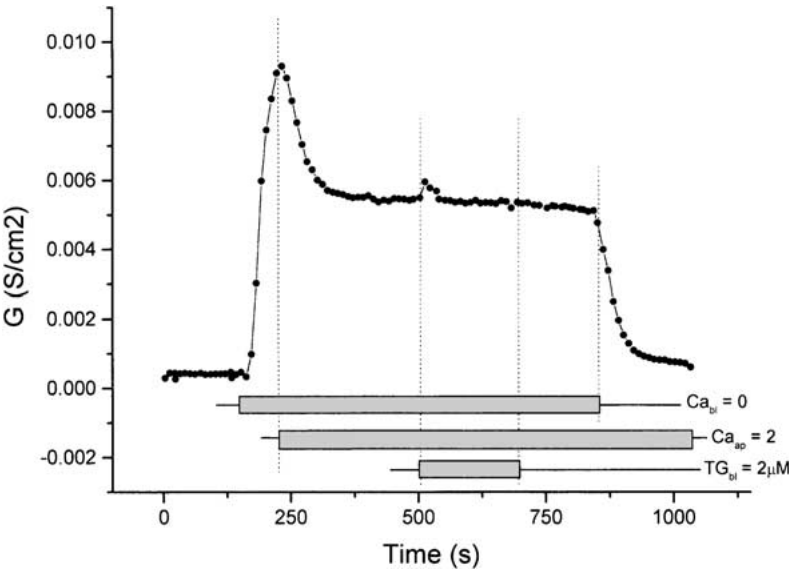


Fig. 7. Representative experiment (of a group of 5) performed in A6 cell monolayers on the effect of thapsigargin (TG) added to the basolateral solution (2 μM) when TJs were kept in the metastable condition, as already described. Monolayers were loaded with the Ca^{2+} chelator BAPTA-AM, a cell-permeant ester (10 μM), on both sides for 20 min prior to $Ca^{2+}{}_{bl}$ removal.

partial recuperation of *G*. In no circumstance, however, a decline of *G* was detected as the first response to TG addition, indicating that a rise of $Ca^{2+}{}_{cell}$, in consonance with the Ca^{2+} ionophore results, never caused in our experiments an increase of the TJ seal. Mean values are presented in Fig. 3 columns *F* and *G*. When the Ca^{2+} ionophore A23187 was tested superimposed on thapsigargin, its effect was markedly reduced or abolished both in frog urinary bladders and A6 cell monolayers (*results not shown*).

Experiments with BAPTA-AM

To ascertain the contribution of cytosolic Ca^{2+} concentration increase on the rise of *G* induced by thapsigargin, A6 cell monolayers were loaded with the Ca^{2+} chelator BAPTA-AM, a cell-permeant ester (10 μM) on both sides for 20 min, as previously used (Lacaz-Vieira, 1997). In a group of 5 experiments, 4 showed no significant response to thapsigargin and 1 a much smaller response as compared with the experiments where the chelator was absent. Fig. 7 is a representative experiment. These experiments also show that buffering cell Ca^{2+} concentration with BAPTA-AM does not affect at all TJ closure in response to Ca^{2+} return to the basolateral solution. Mean values are presented in Fig. 3 column *H*.

TJ Oscillations Are Transiently Halted in Response to a Rise of Cell Ca^{2+} Concentration

TJ permeability in the frog urinary bladder, in the course of an FCSA, may oscillate when Ca^{2+} is present in the apical solution, as shown previously (Lacaz-Vieira, 2000; Kassab, Jr. et al., 2002). These oscillatory TJ behavior is characterized by oscillations of tissue electrical conductance (*G*), and was interpreted as resulting from oscillations of a negative feedback loop in which the control signal is the Ca^{2+} concentration at *zonula adhaerens*; the receptors are the Ca^{2+}-binding sites of *zonula adhaerens* and the effector, the TJs (Kassab, Jr. et al., 2002).

In the present protocol, oscillations were induced in the frog urinary bladder as described (Lacaz-Vieira, 2000) and the Ca^{2+} ionophore (A23187) was added to the Ca^{2+}-containing apical solution at 1 μM concentration leading, as expected (Jovov et al., 1994), to a rise of cell Ca^{2+} concentration. As shown in Fig. 8 this maneuver induces a transient halt in the oscillations, which reappear after minutes with a smaller amplitude.

Discussion

The central focus of the present study is to critically evaluate how changes of extracellular Ca^{2+} concentration ($Ca^{2+}{}_{ext}$) modulate TJ permeability in short-duration experiments in which later regulatory processes are minimized. As seen in the Introduction, a reduction of $Ca^{2+}{}_{ext}$ leads to an increase of TJ permeability in natural epithelia, as well as in monolayers of cells in culture, and a subsequent $Ca^{2+}{}_{ext}$ increase has the opposite effect, including triggering the formation of the junctional complex in cultured cell monolayers freshly plated.

Various studies indicate that the effects of changes of $Ca^{2+}{}_{ext}$ on the TJs result from a direct effect of Ca^{2+} upon the Ca^{2+} binding sites on the extracellular cell surface (Gumbiner et al., 1988; González-Mariscal et al., 1990; Contreras et al., 1992a, 1992b; Gorodeski Jin & Hupfer, 1997). In contrast, others argue that these effects result from changes of $Ca^{2+}{}_{cell}$ that are subsequent to changes in $Ca^{2+}{}_{ext}$ (Bhat et al., 1993; Jovov et al., 1994; Stuart et

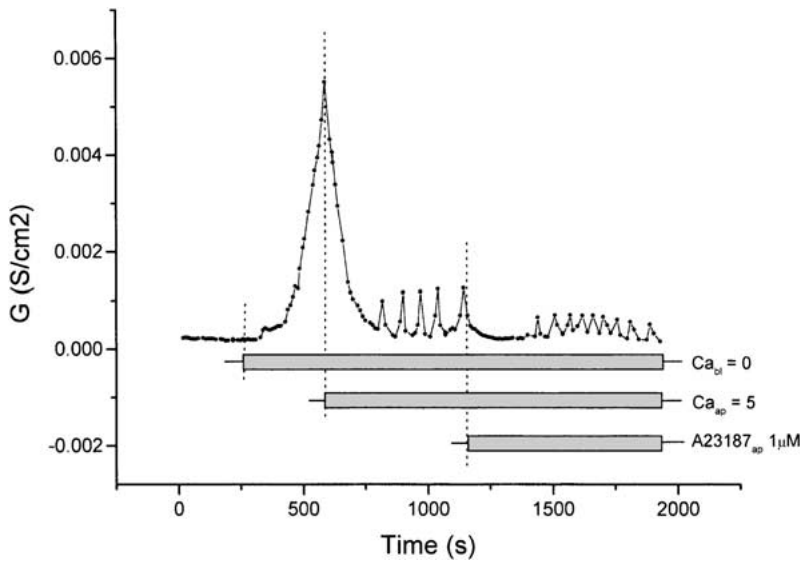


Fig. 8. Representative experiments (of a group of 4) performed in frog urinary bladders on the effect of the Ca^{2+} ionophore A23187 on TJ oscillations.

al., 1994; Stuart et al., 1996). In view of these discrepancies, we decided to evaluate in two different preparations, the frog urinary bladder and A6 cell monolayers, by means of the fast Ca^{2+} switch assay (FCSA) the effects of increasing $Ca^{2+}{}_{cell}$ upon tissue electrical conductance (G), which is an accurate parameter of TJ permeability when Na^+ is absent from the apical bathing fluid (Wills & Millinoff, 1990; Jovov et al., 1994; Lacaz-Vieira & Kachar, 1996). Two different procedures were used to promote increase of $Ca^{2+}{}_{cell}$, the use of the Ca^{2+} ionophore A23187 (Reed & Lardy, 1972) and of thapsigargin, a specific inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (Stuart et al., 1996; Jan et al., 1999), both inducing coherent results in A6 cell monolayers as well as in frog urinary bladders.

A particular experimental condition, in which the TJs are in a partially opened metastable condition, obtained by opening the TJs through Ca^{2+}_{cl} removal followed by blocking the opening process with $Ca^{2+}{}_{ap}$, allowed us to test the effects of increasing $Ca^{2+}{}_{cell}$ on TJ dynamics. In this condition TJs remain partially open for a reasonably long period of time. This is an attractive situation since the TJs can evolve to further opening or closing according to the challenge they are submitted to. In this metastable condition we tested the effects of increasing $Ca^{2+}{}_{cell}$ induced by the Ca^{2+} ionophore or by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor.

In all tested experimental conditions, both in A6 cell monolayers and frog urinary bladders, the first response to a rise of $Ca^{2+}{}_{cell}$ was always an increase of G; in no case a decrease of G was observed. This increase of G, normally transient, is followed by a recuperation of G to levels close to those prior to the use of A23187 or thapsigargin. This behavior is in sharp contrast with the response induced by returning Ca^{2+} to the basolateral solution, a maneuver that always causes a sharp decrease of G, starting a few seconds after $Ca^{2+}{}_{bl}$ addition, and is followed by a total recovery of the TJ seal. These results strongly support the interpretation that the effect of $Ca^{2+}{}_{ext}$ on the TJs might result directly from its interaction with extracellular Ca^{2+} binding sites, the E-cadherin molecules mostly located in the *zonula adhaerens* (González-Mariscal et al., 1990; Contreras et al., 1992a, 1992b; Gorodeski et al., 1997; Gumbiner et al., 1988). Our data are in conflict with observations showing that TJ closure in response to a rise of $Ca^{2+}{}_{bl}$ is triggered by a rise of $Ca^{2+}{}_{cell}$ (Jovov et al., 1994). These authors observed in A6 cell monolayers exposed to a lower $Ca^{2+}{}_{bl}$ that addition of A23187 to a Ca^{2+}-containing apical solution caused a biphasic response similar to the ones that we have observed with Ca^{2+} ionophore or thapsigargin. Their initial decrease of tissue electrical resistance (which is equivalent to our increase of G), which soon reversed with a significant recovery of tissue electrical resistance, was interpreted as resulting from an increase of apical membrane conductance due to the ionophore. Thus, after suitable corrections, the authors (Jovov et al., 1994) concluded that the rise of $Ca^{2+}{}_{cell}$ by the ionophore led to a complete recovery of the TJ seal, as evaluated by a total recovery of R_t and, therefore, that the action of Ca^{2+} leading to TJ closure was intracellular. Our results with thapsigargin and with the Ca^{2+} ionophore A23187 merit, however, a different interpretation. Since in our control condition, with the TJs closed, addition of A23187 to the apical solution, both in the presence or absence of $Ca^{2+}{}_{ap}$, did not cause a significant increase of G (Figs. 1 and 2), this warrants us to rule out that the ionophore, in the concentration used in our experiments, would per se increase tissue electrical conductance by increasing apical membrane conductance. Another discrepancy between our data and theirs (Jovov et al., 1994) is the

time course of G response when $Ca^{2+}{}_{bl}$ is lowered. These differences might in part result from the types of chamber used. In our experiments, both in frog urinary bladders and A6 cells, it took only 1 to 3 min for G to start lowering, while in their experiments, R_t remained unchanged for nearly 30 min. Our chambers allow a fast renewal of the solutions in contact with both tissue surfaces due to a continuous flow of fresh solution directed perpendicularly to these surfaces. In addition, in our experiments $Ca^{2+}{}_{bl}$ was totally removed by using a Ca^{2+}-free solution. The argument the authors (Jovov et al., 1994) used in favor of an intracellular action of Ca^{2+} on the TJs was that the time course of changes in $Ca^{2+}{}_{cell}$ in response to changes of $Ca^{2+}{}_{bl}$ were faster than those of R_t. They interpreted their results suggesting that the basolateral membrane is highly permeable to Ca^{2+} in a way that changes in $Ca^{2+}{}_{bl}$ results in changes of $Ca^{2+}{}_{cell}$ so that a decrease in $Ca^{2+}{}_{cell}$ precedes TJ opening and that closing of TJ follows increase of $Ca^{2+}{}_{cell}$.

Chelation of $Ca^{2+}{}_{cell}$ with BAPTA-AM markedly attenuated or totally abolished the biphasic response to thapsigargin in A6 cell monolayers indicating that this response might indeed be caused by a rise of $Ca^{2+}{}_{cell}$. However, chelation of $Ca^{2+}{}_{cell}$ does not prevent TJ closure in response to return of Ca^{2+} to the basolateral solution, which is another strong argument in favor of a predominantly extracellular action of Ca^{2+} on TJ dynamics. A different tissue response observed in frog urinary bladders to a rise of $Ca^{2+}{}_{cell}$ is a transient interruption of TJ oscillations (Fig. 8) induced by addition of A23187 to a Ca^{2+}-containing apical solution. In these experiments, in which the TJ permeability is close to the control initial value, the biphasic response normally elicited by raising $Ca^{2+}{}_{cell}$ is not observed, only interruption of oscillations is detected.

The existence of a biphasic response and interruption of oscillations clearly indicate that a rise of $Ca^{2+}{}_{cell}$, despite of not causing a seal of the TJs, both in the frog urinary bladder and in A6 cell monolayers, affect TJ dynamics.

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