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Tight Junction Dynamics: Oscillations and the Role of Protein Kinase C

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Abstract. The present study aimed to characterize the role of protein kinase C (PKC) on the dynamics of tight junction (TJ) opening and closing in the frog urinary bladder. The early events of TJ dynamics were evaluated by the fast Ca^{++} switch assay (FCSA), which consisted in opening the TJs by removing basolateral Ca^{++} ($[Ca^{++}]_{bl}$), and closing them by returning $[Ca^{++}]_{bl}$ to normal values. Changes in TJ permeability can be reliably gauged through changes of transepithelial electrical conductance (G) determined in the absence of apical Na^{+}. The FCSA allows the appraisal of drugs and procedures acting upon the mechanism controlling the TJs. The time courses of TJ opening and closing in an FCSA were shown to follow single exponential time courses. PKC inhibition by H7 (100 μM) caused a reduction of the rate of junction opening in response to removing $[Ca^{++}]_{bl}$, without affecting junction closing, indicating that PKC is a key element in the control of TJ opening dynamics in this preparation. H7 at 250 μM almost completely inhibits TJ opening in response to basolateral Ca^{++} withdrawal. Subsequent H7 removal caused a prompt inhibition release characterized by a sharp G increase which, however, once started cannot be stopped by H7 reintroduction, Ca^{++} being necessary to allow TJ recovery. A step rise of apical Ca^{++} concentration ($[Ca^{++}]_{ap}$) causes a reduction of the rate of TJ opening in a FCSA, an effect that is believed to be mediated by apical Ca^{++} entering the open TJs. The specific condition of having Ca^{++} only in the apical solution and the TJs located midway between the Ca^{++} source (apical solution) and the Ca^{++}-binding sites presumably located at the *zonula adhaerens*, might configure a situation in which a control feedback loop is set up. A rise of $[Ca^{++}]_{ap}$ during the phase of G increase in an FCSA causes a transient recovery of G followed by a subsequent escape phase where G increases again. Oscillations of G also appear in response to a rise of apical Ca^{++}. Both escape and oscillations result from the properties of the TJ regulatory feedback loop. In conclusion, the present results indicate that PKC plays a key role in TJ opening in response to extracellular Ca^{++} withdrawal without major effect on the reverse process. In addition, PKC inhibition by H7 not only prevents TJ opening in response to basolateral Ca^{++} removal but induces a prompt blockade of TJ oscillations induced by apical Ca^{++}, oscillations which reappear again when H7 is removed.

Key words: Tight junction — Protein kinase C — Calcium — Oscillations — Paracellular conductance — Feedback control

Introduction

Extracellular Ca^{++} is essential for cells to maintain intercellular contacts and for stability of cell junctions. A number of studies in natural epithelia and in cell culture monolayers show that when extracellular Ca^{++} is lowered, junctions are weakened and the tight junctions (TJ) become leaky; the return of Ca^{++} to tissues that had their TJs opened by extracellular Ca^{++} removal causes recovery of the TJ seal (Sedar & Forte, 1964; Hays et al., 1965; Galli et al., 1976; Meldolesi et al., 1978; Pitelka et al., 1983; Palant et al., 1983; Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997; Lacaz-Vieira et al., 1999), or induce the development of new TJs in cell cultures in confluence (Martinez-Palomo et al., 1980; Cereijido et al., 1980; Cereijido et al., 1981). While the effect of extracellular Ca^{++} on TJ dynamics is well characterized, the complete sequence of events that links the interaction of Ca^{++} with the Ca^{++}-binding sites of *zonula adhaerens*, leading to the closing of TJs, is far from being completely understood, despite a large body of information on this subject. The cell adhesion molecule E-cadherin

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(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 domain that modulates the formation and maintenance of the epithelial junctional complex (Gumbiner et al., 1988). Ca^{2+} determines the conformation of E-cadherin and stabilizes the molecule in its adhesive state (Ringwald et al., 1987). TJs are highly complex structures with delicate cellular mechanisms modulating junction dynamics, which comprises recognition sites, signaling systems and intracellular events triggered by Ca^{++} acting on the extracellular aspects of the cells. The interaction of Ca^{2+} with E-cadherin is transduced to the TJs by a cascade of reactions involving phospholypase C, G proteins, protein kinase C (PKC) and calmodulin (Balda et al., 1991, 1993). The role of PKC is further supported by other observations (Knight et al., 1988; Nigam et al., 1991; Citi, 1992). Antibodies to E-cadherin block junction formation and diC8 counteracts this effect, supporting that PKC is in the route activated by E-cadherin mediated cell-cell adhesion (Balda et al., 1993). In MDCK monolayers, PKC inhibition prevented TJ dissociation induced by low extracellular Ca^{++} (Citi, 1992) and its activation increases the permeability of rat hepatocyte TJs (Nathanson et al., 1992). The role of PKC on TJ dynamics is in consonance with its role upon other types of intercellular contacts (Sheu et al., 1989; Winkel et al., 1990). Activation of PKC by phorbol esters (Ojakian, 1981; Mullin & O'Brien, 1986) or diacylglicerols (Mullin & McGinn, 1988) resulted in an increase of TJ permeability in monolayers of LLC-PK1 or MDCK cells with intact TJs. A cellular variability in the development of TJ after activation of PKC has been described in different cell systems (Ellis et al., 1992), indicating that the details of the actual mechanisms regulating the TJs are yet poorly understood. Vinculin phosphorylation by protein kinase C is a crucial step in the correct assembly of the epithelial junctional complex (Perez-Moreno et al., 1998). A clear picture of the effects of drugs that affect cell signaling systems upon the TJs is still lacking due to differences in experimental protocols and preparations. For example, activators or inhibitors were tested on mature epithelial membranes (natural or artificial monolayers), during the reorganization of isolated cells into an epithelial membrane (several hours of experiments) (Ellis et al., 1992), during the Ca^{++} switch assay (several hours) (González-Mariscal et al., 1985) or during the fast Ca^{++} switch assay (minutes) (Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997; Lacaz-Vieira et al., 1999). The role of intracellular calcium on TJ dynamics is a disputed subject (González-Mariscal et al, 1990; Jovov et al., 1994; Gorodeski et al., 1997). Cell Ca^{++} plays a facilitatory role in the reassembly of the TJ damaged by ischemic insults, appearing to be necessary for the dissociation of TJ-cytoskeletal complexes, thus permitting functional TJ reassembly and paracellular permeability barrier recovery (Ye et al., 1999). The use of protocols like the "calcium switch" (González-Mariscal et al., 1985), the "ATP depletion-repletion" model (Tsukamoto & Nigam, 1997) and the fast Ca^{++} switch assay (FCSA) (Lacaz-Vieira & Kachar, 1996) allowed insights regarding the dynamics of TJs.

The present study focuses on the dynamic aspects of TJ regulation. We use a fast Ca^{++} switch assay (FCSA) to study the early events related to TJ opening and closing in response to basolateral Ca^{++} manipulations. This assay allowed a dynamic study of TJ opening and closing and of the role of PKC on these responses. It was also shown that TJs are able to oscillate when properly tested in a FCSA with Ca^{++} present only in the apical bathing medium.

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) at a dose of 1 ml/100 gr. 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. 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 (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-Ringer: NaCl 115, $KHCO_3$ 2.5, and $CaCl_2$ 1.0. The apical bathing fluids were simple salt solutions, nonbuffered, 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). The apical solution was KCl 75 mM in order to eliminate Na^+ from this solution, ruling out any 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^{++} concentration increase or decrease in this region in response to changes of Ca^{++} 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 3M KCl electrodes and 3M 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, Foster City, CA) and stored in a computer for further processing.

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO). H7: 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine.

Statistics

The results are presented as mean ± SE. 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^{++}-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 a FCSA.

Abbreviations and Conventions

TJ: Tight junction

FCSA: Fast Ca^{++} switch assay.

I: Clamping current, in $\mu A\ cm^{-2}$. 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 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.

The concentrations of ions and substances in the bathing solutions are specified as X_y, where X indicates the substance or ion and *y* the bathing solution (apical or basolateral), and expressed in mM if not otherwise stated. τ_1 and τ_2 are the rate constants of TJ opening and closing (in sec) in response to a FCSA.

Results

Effect of Protein Kinase Inhibition on the Kinetics of TJ Opening and Closing

These experiments were carried out to evaluate the effect of protein kinase C (PKC) on the kinetics of TJ opening and closing in the frog urinary bladder. In the absence of apical Na^+, *G* is a reliable evaluation of paracellular electrical conductance which mainly reflects the contribution of the TJ barrier (Lacaz-Vieira & Kachar, 1996). Control experiments (to be compared later to conditions in the presence of H7, a PKC inhibitor) were carried out to characterize bladder electrical conductance (*G*) response to a fast Ca^{++} switch assay (FCSA), which consisted in triggering the opening of TJs by removing Ca^{++} from the basolateral solution followed by the closing in response to a subsequent reintroduction of Ca^{++} into this solution (Lacaz-Vieira, 1997). In the control condition, urinary bladders were bathed on the apical side by a nominally Ca^{++}-free KCl 75 mM solution, to demote the contribution of transcellular Na^+ pathway to the overall tissue electrical conductance, and on the basolateral side by NaCl Ringer's solution. Tissues were previously equilibrated for several minutes in these solutions, showing a stable *G* of $8.11 \times 10^{-5} \pm 6.42 \times 10^{-6}$ S/cm^2 ($n = 22$). After an average delay of 103.0 ± 9.2 sec ($n = 22$), *G* begins to increase at a fast growing rate in response to basolateral Ca^{++} removal. To standardize the experimental protocol the process of junction opening was always halted, when *G* reached values of the order of 5×10^{-3} S/cm^2, by returning Ca^{++} to the basolateral solution leading to a complete recovery of *G* in all cases. The time courses of *G* increase and decrease in response to an FCSA followed single exponential time courses, that could be characterized by single time constants. The increase of *G* in response to basolateral Ca^{++} removal followed an exponential growth according to $G = G_O + A_1 \exp((t - t_O)/\tau_1)$, where G_O and t_O are respectively the offset values of *G* and *t*, A_1 is the amplitude parameter, and τ_1 the time constant of the exponential growth, which had an average value of 28.5 ± 3.1 sec ($n = 13$). The decrease of *G* in response to Ca^{++} return to the basolateral solution followed a first order exponential decay, according to: $G = G_O + A_2 \exp\text{-}((t - t_O)\tau_2)$, where G_O and t_O are offset values of *G* and *t*, A_2 is the amplitude parameter, and τ_2 the time constant of the first order exponential decay, which had an average value of 56.7 ± 6.4 sec ($n = 13$). Fig. 1*A* and *B* show, respectively, for a representative experiment, *G* response to an FCSA as well as the exponential fitting to both the increase and the decrease of *G*.

For the test group in which H7 was used to block PKC, each experiment started with an initial control FCSA carried out to determine the untreated tissue response pattern. Then, the PKC inhibitor, H7, at different concentrations (10 to 250 μM) was added to the inner solution. A period of equilibration followed, and subsequent FCSAs were carried out at successive time intervals. An effect of H7 started to be clearly noticed at 50 μM and reached a maximum effect at 250 μM. A representative case of a group of 8 experiments where H7 was

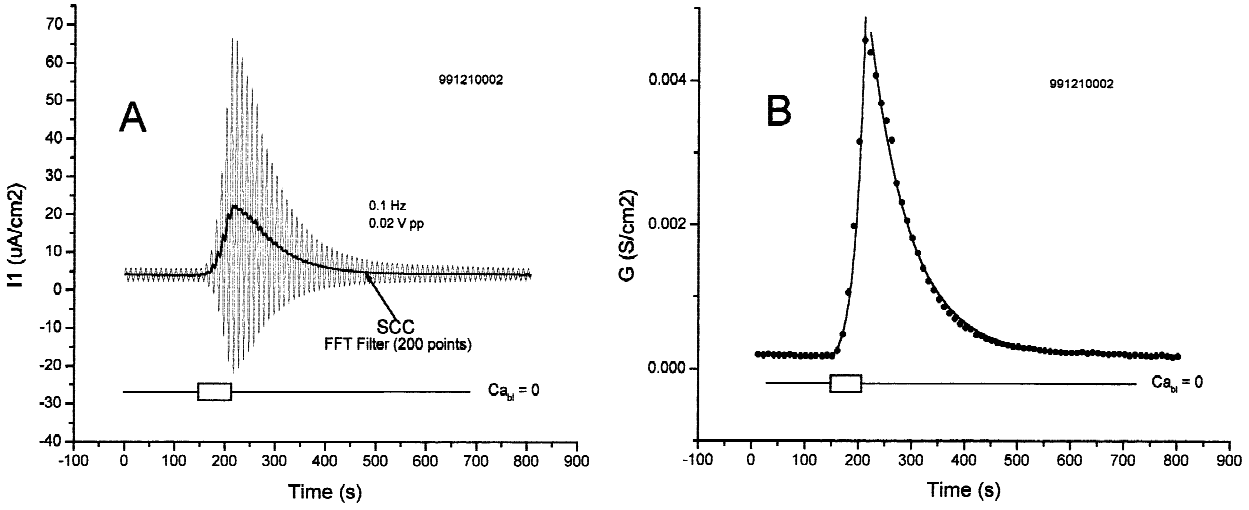

Fig. 1. Representative experiment (of a group of 8) of the time course of tissue electrical conductance (*G*) increase and decrease in response to a fast Ca^{++} switch assay (FCSA) which consisted in removing Ca^{++} from the basolateral solution, causing an increase of *G* due to tight junction (TJ) opening, and a subsequent Ca^{++} reintroduction, causing a decrease of *G* due to TJ resealing. The apical solution when not otherwise indicated was KCl 75 mM and the basolateral, NaCl-Ringers solution. The gray rectangles indicate the periods in which there were changes in concentration which are then specified in the figure at the right end of the lines passing through the rectangles. When units are not specified, the concentrations are in mM; (*A*) shows a plot of the clamping current as a function of time. The clamping voltage followed a sine wave of 0.02 V peak to peak and 0.1 Hz to allow calculation of *G*. The short-circuit current (SCC) was obtained by applying a fast Fourier transform filter to the clamping current (*see* Materials and Methods); (*B*) shows the time course of *G* increase and decrease in response to an FCSA. As can be seen, both the increase and the decrease of *G* closely follow single exponential time courses.

used at 100 μM is shown in Fig. 2. As can be seen, H7 (100 μM) caused a marked reduction of the speed of *G* increase in response to basolateral Ca^{++} removal (increase of the time constant, τ_1), without appreciably effecting the time course of *G* recovery (τ_2) in response to Ca^{++} return to the basolateral solution. At this concentration, the effect of H7 builds up progressively and normally takes about 30 to 60 min to attain full effect. With higher H7 concentrations these times are appreciably shortened. In contrast, H7 removal causes a rapid inhibition decline, full recovery taking place in a few minutes. In addition, an overshoot effect is observed, characterized by an increase of the rate of *G* increase (decrease of τ_1) in response to basolateral Ca^{++} removal, the rate rising above the value measured previously to H7 addition, as shown in Fig. 3 where mean values are shown for the time constants of TJ opening (τ_1) and closing (τ_2) evaluated in response to a FCSA for three experimental groups: (i) control, (ii) H7 100 μM/110 min and (iii) 8 min after H7 removal. When higher H7 concentrations were used, a more drastic effect on the rate of *G* increase in response to basolateral Ca^{++} removal was observed. At the maximum concentration used (250 μM) a marked, almost complete inhibition of TJ opening in response to basolateral Ca^{++} removal was observed. In this case TJs remain closed in the total absence of external Ca^{++} (both apical and basolateral). At this H7 concentration, bladder may remain 10 min (maximum period of observation) in a Ca^{++}-free basolateral solution without showing any appreciable increase of *G* (Fig. 4). A very characteristic aspect of H7 inhibition of TJ opening in response to a Ca^{++}-free basolateral solution (apical solution is always Ca^{++} free) was that drug removal caused an almost immediate (delay of few seconds) and sharp increase of *G*. As soon as *G* starts to increase, reintroduction of H7 at the same concentration into the basolateral solution normally caused a reduction of the speed of *G* increase, without, however, halting the process (Fig. 4).

Nevertheless, when Ca^{++} is returned to the basolateral solution (in the presence of H7), there is an almost immediate stop of *G* increase, leading to a complete recovery of the TJ seal, as it does in the control condition. Even for the maximum H7 concentration used (250 μM) no impairment was observed on *G* recovery in response to basolateral Ca^{++} return, indicating that PKC plays an important role only on TJ opening in response to basolateral Ca^{++} removal without appreciable effect on junction recovery.

Effects of Apical Ca^{++} on the Fast Ca^{++} Switch Assay: Escape and Oscillations of *G*

These experiments were carried out in order to test the role of apical Ca^{++} on TJs previously opened by the removal of basolateral Ca^{++}. In a previous study (Lacaz-Vieira & Kachar, 1996) it was shown that apical Ca^{++}

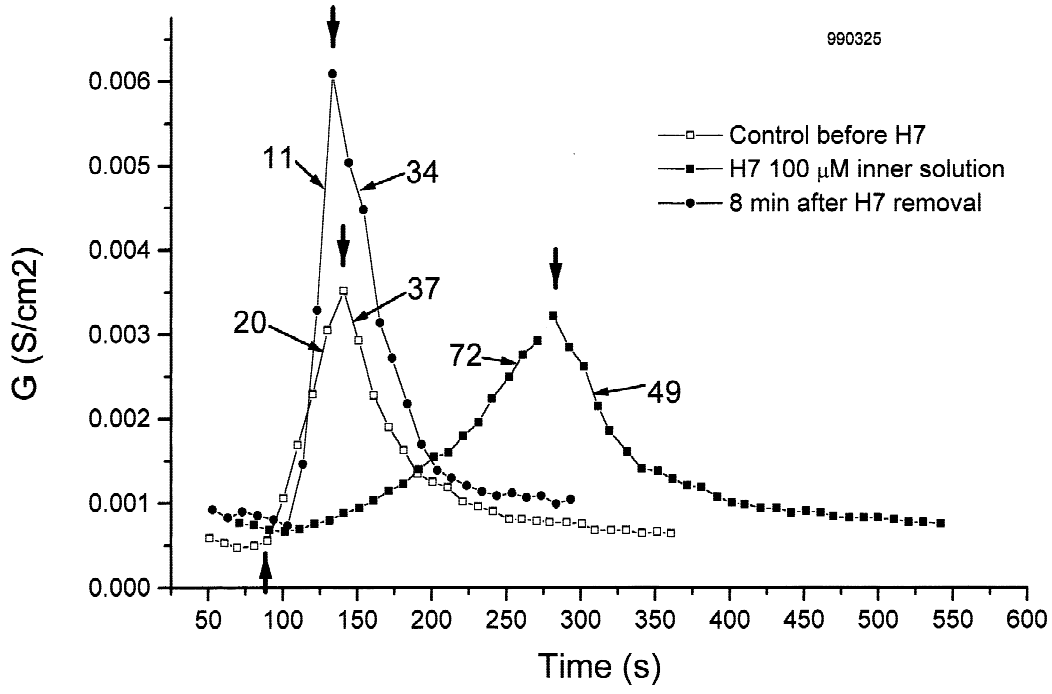

Fig. 2. Representative experiment of the effect of the protein kinase inhibitor (H7) added to the inner solution at 100 μM concentration on the time course of *G* increase and decrease in response to a FCSA, which consisted in opening the TJs by removing Ca^{++} from the basolateral solution (upward arrow), and subsequently closing the junctions by Ca^{++} reintroduction to this solution (downward arrows). The apical solution was KCl 75 mM and the basolateral, NaCl-Ringer's solution. The numbers are individual values of the time constants (in seconds) of *G* increase and decrease in response to a FCSA.

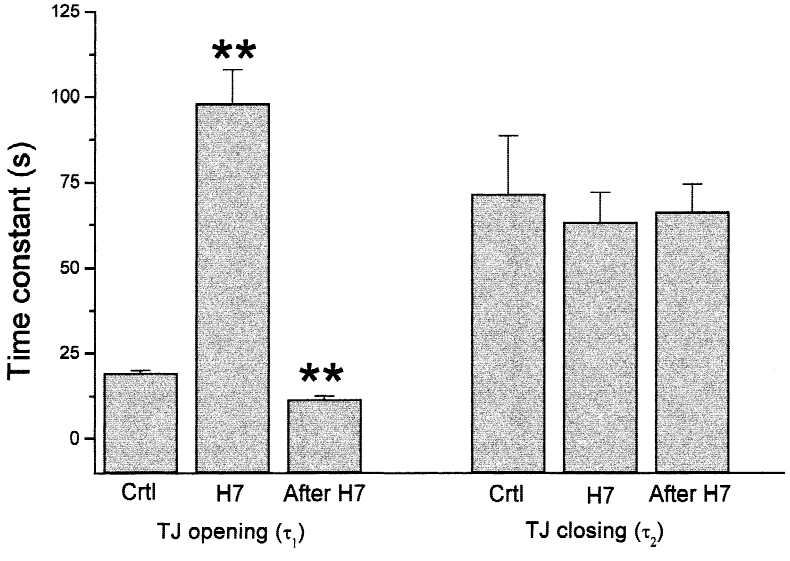

Fig. 3. Effect of the protein kinase inhibitor (H7) added to the inner solution at 100 μM concentration on the time course of *G* increase and decrease in response to an FCSA, which consisted in opening the TJs by removing Ca^{++} from the basolateral solution and subsequently closing the junctions by Ca^{++} reintroduction into this solution (as shown in Fig. 2 for a representative experiment). Mean values for the time constants for TJ opening (τ_1) and TJ closing (τ_2) measured in the control condition before H7 (Ctrl), 110 min after H7 100 μM was added to the inner solution (H7), and 8 min after H7 removal from the inner bathing solution (After H7). For τ_1, the difference between Ctrl and H7 ($P < 0.001$, paired *t* test) and for Ctrl and After H7 ($P < 0.001$, paired *t* test), $n = 8$.

may induce the closure of the TJs (opened by basolateral Ca^{++} removal) by entering these open TJs. The rationale of the present experiments is that TJs limiting diffusion of molecules and ions would also restrict diffusion of Ca^{++} from the apical solution into the *zonula adhaerens* where the Ca^{++} binding sites, the E-cadherin molecules, that control the TJs are located (Gumbiner et al., 1988). Thus, it is expected that apical Ca^{++} diffusing through open TJs might reach the Ca^{++}-binding sites at the *zonula adhaerens* inducing TJ closure. Junction sealing, in turn, would reduce the flow of Ca^{++} into *zonula adhaerens.* As Ca^{++} continuously diffuses from the *zonula adhaerens*

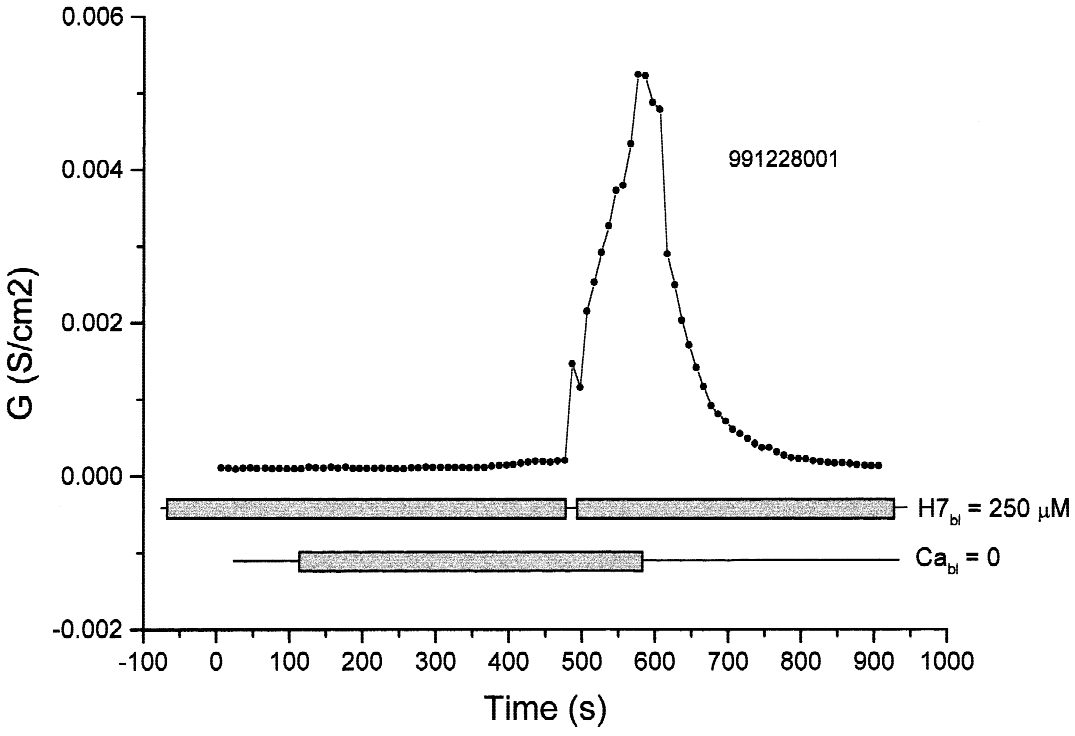

Fig. 4. Representative experiment (of a group of 6) of the effect of the protein kinase inhibitor (H7) added to the inner solution at 250 μM concentration on the time course of *G* increase and decrease in response to an FCSA, which consisted in opening the TJs by removing Ca^{++} from the basolateral solution and subsequently closing the junctions by Ca^{++} reintroduction into this solution. The apical solution when not otherwise indicated was KCl 75 mM and the basolateral, NaCl-Ringers solution. The gray rectangles indicate the periods in which there were changes in concentration which are then specified in the figure at the right end of the lines passing throughout the rectangles. When units are not specified, the concentrations are in mM. The bladder was exposed to the inhibitor for 15 min. Then Ca^{++} was removed from the basolateral solution without inducing the characteristic increase of *G* observed in control tissues, indicating that the TJs remained closed. After 6 min of a Ca^{++}-free basolateral solution, H7 was removed from the basolateral solution causing a sharp increase of *G* with a very short delay. Reintroduction of H7 to the basolateral solution (after 30 sec) slowed down the rate of *G* increase but did not halt the process of *G* increase. Finally, return of Ca^{++} to the basolateral medium induced full recovery of the TJ seal.

into the lateral spaces, and ultimately into the inner solution (where $[Ca^{++}]_{bl} = 0$ mM), this would cause a drop of Ca^{++} concentration at the Ca^{++} binding sites, leading to a subsequent opening of the TJs. Then, cycles of TJ openings and closings might be expected to occur and be characterized by oscillations of *G*. The experimental demonstration of these oscillations would be a dynamic confirmation that the Ca^{++} sites that control TJ are mainly located at the *zonula adhaerens* and that diffusion of apical Ca^{++} through the TJs indeed modulates the TJs. In addition, procedures and drugs that would affect these oscillations might be an indication that they somehow interfere with cell pathways involved with the control of the TJs.

To characterize the effects of apical Ca^{++} on TJ dynamics, pulses of apical Ca^{++} were given to raise apical Ca^{++} concentration while the TJs were in the opening phase of a fast Ca^{++} switch assay (FCSA). The protocol consisted in opening the TJs by removing Ca^{++} from the basolateral solution followed by adding Ca^{++} to the apical solution. Figure 5 shows a sequence of experiments performed in a single piece of bladder displaying bladder electrical conductance (*G*) in response to a FCSA. Figure 5*A* is a control FCSA performed in the absence of apical Ca^{++}. When a pulse of apical Ca^{++} is given during TJ opening in response to basolateral Ca^{++} removal, it triggers a partial transient TJ closing, characterized by a transient reduction of *G*, a process that escapes afterwards, eventually reaching stabilization of *G*. The stabilization level depends on the Ca^{++} concentration attained in the apical bathing fluid. Thus, in Fig. 5*B* apical Ca^{++} concentration was raised to 1 mM, inducing a prompt stop in the process of *G* increase, a short recovery period followed by an escape phase in which *G* increases again, while in Fig. 5*C* the apical Ca^{++} concentration was raised to 5 mM, inducing a much larger recovery which then escapes at a slower rate than in Fig. 5*B*. Frequently, oscillations of *G* may develop in response to addition of Ca^{++} to the apical solution, as shown in Figs. 5*C* and 6*A*. When Ca^{++} is already present in the apical solution prior to an FCSA, the rise of *G* develops at a slower rate as compared to the apical Ca^{++}-free condition, and the in-

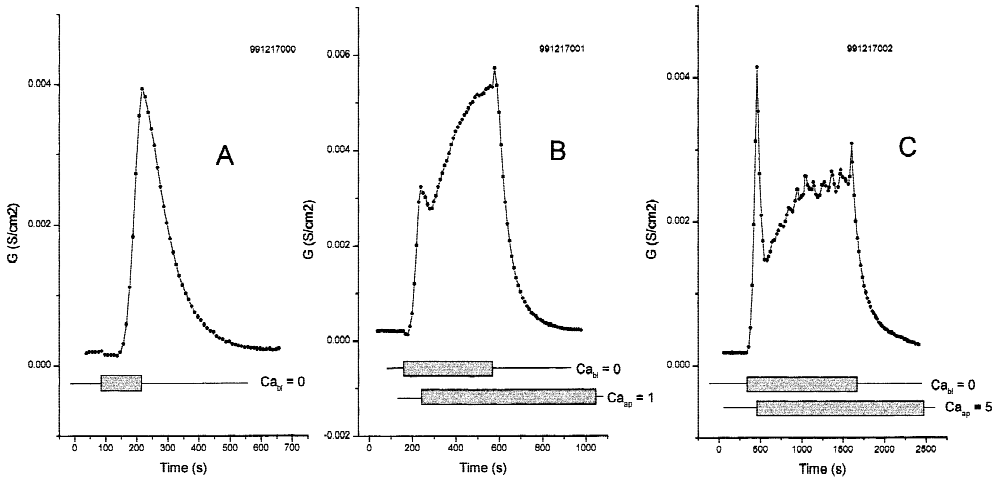

Fig. 5. Representative experiment (of a group of 12) performed in a single bladder of the effect of rising apical Ca^{++} concentration on the time course of junction opening, evaluated by changes of transepithelial electrical conductance (*G*) in response to a fast Ca^{++} switch assay (FCSA), which consisted in opening the TJs by removing Ca^{++} from the basolateral solution and subsequently closing the junctions by Ca^{++} reintroduction into this solution. The apical solution when not otherwise indicated was KCl 75 mM and the basolateral, NaCl-Ringers solution. The gray rectangles indicate the periods in which there were changes in concentration which are then specified in the figure at the right end of the lines passing through the rectangles. When units are not specified, the concentrations are in mM. (*A*) Control tissue response to an FCSA. (*B*) Effect of rising apical Ca^{++} concentration to 1 mM during the course of junction opening. Addition of apical Ca^{++} halts *G* increase almost immediately, causes a partial transient *G* recovery which is followed by a escape phase characterized by subsequent increase of *G* at a slower rate than before. Return of Ca^{++} to the basolateral medium halts junction opening causing a complete recovery of the TJ seal. (*C*) Effect of rising apical Ca^{++} concentration to 5 mM in the course of junction opening. A more marked transient recovery of *G* is observed. In addition, clear oscillations of *G* during the escape phase can be observed as *G* evolves to a stationary condition. Finally, full recovery is induced by the return of Ca^{++} to the basolateral solution.

crease of *G* may also be accompanied by oscillations (Fig. 6*B*).

The partial recovery of *G* induced by apical Ca^{++}, the escape phase and the oscillations can all be interpreted based on the premises put forward in the beginning of this section related to a feedback control mechanism (*see* Discussion).

Role of PKC on TJ Oscillations

As seen before, PKC plays a significant role on the process of TJ opening, since its inhibition by H7 markedly affects the rate of TJ opening in an FCSA without affecting the reverse process. Therefore, because PKC is a key element in the control of TJs, its inhibition would be expected to affect the balance of forces that determine the TJ state and, consequently, through the feedback loop, the oscillations of the TJ barrier. Figure 7 is a representative example of the effect of PKC inhibition upon TJ oscillations. Oscillations were induced by adding apical Ca^{++} (5 mM) during the process of TJ opening in a FCSA. When *G* attained a stable value and oscillations were clearly seen, H7 (250 μM) was added to the basolateral solution inducing an almost immediate block of the oscillations and a marked recovery of the TJ seal, as indicated by a reduction of *G*. Subsequently, when *G* had leveled off, H7 removal caused a sharp response characterized by an increase of *G* and, again, the establishment of oscillations. This same behavior can be induced again when a new pulse of H7 is applied to the inner solution.

Discussion

The present study analyzes in the frog urinary bladder, a natural epithelia, the early events of tight junction (TJ) opening and closing. The dynamics of TJ machinery were assessed by the fast Ca^{++} switch assay (FCSA) (Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997; Lacaz-Vieira et al., 1999), which consists in opening the TJs by making $[Ca^{++}]_{bl} = 0$ mM, followed by a subsequent closing by returning $[Ca^{++}]_{bl}$ to normal values. All experiments were performed in the absence of EGTA in the bathing solutions since the presence of a Ca^{++}-chelating agent that diffuses into the lateral spaces retards the kinetics of TJ opening and closing in response to an FCSA, due to a buffering effect on the Ca^{++} concentration at this compartment. Changes in TJ permeability were gauged through changes of transepithelial electrical conductance (*G*) (Fig. 1) determined in the absence of apical Na^{+}, condition in which *G* is a reliable measure of TJ permeability (Lacaz-Vieira & Kachar, 1996). The FCSA allows assessment of the early kinetic events of TJ opening and closing and the appraisal of the

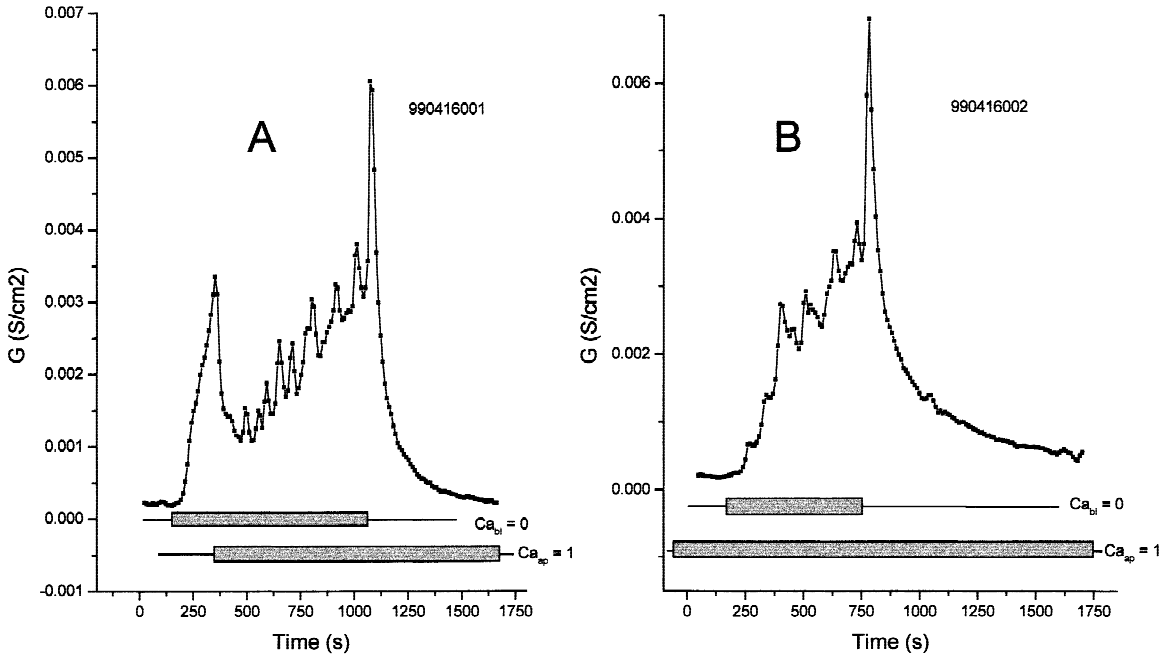

Fig. 6. Representative experiments (of a group of 5 performed in single bladders) of oscillations of *G* induced by apical Ca^{++}. The time course of junction opening was evaluated by the transepithelial electrical conductance (*G*) in response to a fast Ca^{++} switch assay (FCSA), which consisted in opening the TJs by removing Ca^{++} from the basolateral solution and subsequently closing the junctions by Ca^{++} reintroduction into this solution. The apical solution when not otherwise indicated was KCl 75 mM and the basolateral, NaCl-Ringers solution. The gray rectangles indicate the periods in which there were changes in concentration which are then specified in the figure at the right end of the lines passing through the rectangles. When units are not specified, the concentrations are in mM. (*A*) Ca^{++} was added to the apical solution (1 mM) during the course of junction opening. Junction opening was halted and a conspicuous recovery was observed, followed by an escape phase during which *G* increased again. Superimposed oscillations are clearly seen during the escape phase. The return of Ca^{++} to the basolateral medium triggers a peak increase of *G* followed by a recovery process towards full completion. (*B*) Ca^{++} (1 mM) was already present in the apical solution when basolateral Ca^{++} was removed. It can be observed that due to the presence of apical Ca^{++} the rate of *G* increase is markedly slower as compared to the condition of Ca^{++}-free apical solution (*A*). In addition, oscillations of *G* are already present since the beginning of junction opening in response to basolateral Ca^{++} removal due to the presence of apical Ca^{++}. Finally, when Ca^{++} was reintroduced into the basolateral solution a sharp spike of *G* increase is observed followed by a recovery towards completion.

effects of drugs and procedures impinging upon the processes controlling the TJs, while preventing more complex regulatory responses that are involved in long duration experiments. Several sequential runs of junction opening and closing in response to an FCSA could be evoked without any appreciable changes in tissue electrical properties, indicating that the challenges induced by the FCSA were completely reversible. Tight junction opening and closing responses to an FCSA follow single exponential time courses (Fig. 1*B*), characterized by the time constants of *G* increase and decrease, respectively τ_1 and τ_2. This is in consonance with observations in CaSki human cervical cell line where time courses also conformed with simple exponential trends (Gorodeski et al., 1997).

PKC is a key element in TJ regulation as indicated by different lines of investigation (Balda et al., 1991; Citi, 1992; Ellis et al., 1992; Balda et al., 1993; Stenson et al., 1993; Burgstahler & Nathanson, 1995; Citi & Denisenko, 1995; Stuart & Nigam, 1995; Mullin et al., 1998). The present findings show that PKC inhibition affects the kinetics of TJ opening and closing differently. Inhibition of PKC by H7 caused a marked reduction of the rate of junction opening in response to basolateral Ca^{++} withdrawal, without affecting the rate of junction closing in response to Ca^{++} return, indicating that PKC plays a major role only in the process of junction disassembly without contributing to the reverse process (Figs. 2 and 3). In spite of being a potent PKC inhibitor, we cannot rule out that at 250 μM concentration, inhibition of other kinases by H7 may also occur. A complete inhibition of PKC (with 250 μM of H7) leads to a total block of TJ opening in response to basolateral Ca^{++} removal, indicating that the signaling step blocked is critical to junction opening and is not metabolically bypassed in short-term experiments. At this H7 concentration, TJs remain closed in the presence of nominally zero Ca^{++} concentration in both apical and basolateral solutions. With TJ opening totally blocked by H7, removal of the inhibitor causes TJ to open with a delay normally much shorter than that of TJ opening in response to basolateral Ca^{++} removal. It is interesting, however, that despite H7

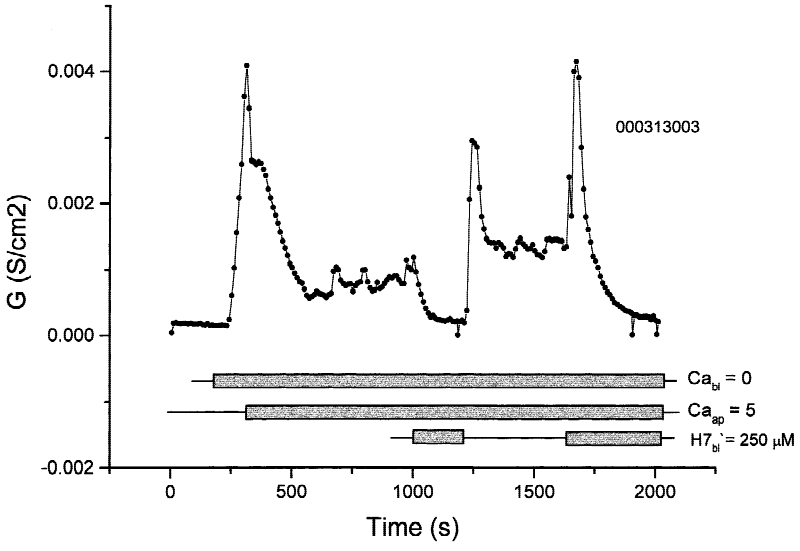

Fig. 7. Representative experiment (of a group of 5 experiments) performed in a single bladder on the effect of pulses of the protein kinase inhibitor (H7, 250 μM) to the inner solution on TJ oscillations (evaluated by *G* oscillations) induced by rising apical Ca^{++} concentration during TJ opening in response to basolateral Ca^{++} removal (*see* Figs. 2 and 3). The apical solution when not otherwise indicated was KCl 75 mM and the basolateral, NaCl-Ringers solution. The gray rectangles indicate the periods in which there were changes in concentration which are then specified in the figure at the right end of the lines passing through the rectangles. When units are not specified, the concentrations are in mM.

being able to maintain the TJs closed in the absence of extracellular Ca^{++} and that its removal causes junction opening, a subsequent return of H7 to the basolateral solution, at the same concentration, is not able to stop the process of junction opening, which can, however, be blocked and reversed by returning Ca^{++} to the inner solution (Fig. 4). Therefore, it can be concluded that, in the total absence of Ca^{++} in the bathing solutions, once TJ opening is triggered by PKC disinhibition, the process cannot be halted by returning H7 to the bathing solution. An interpretation for this fact is still lacking. However if Ca^{++} is present in the apical solution, then addition and removal of H7 triggers TJ closing and opening as judged by reduction and increase of *G* (Fig. 7). Experiments in process in our laboratory in A6 cell monolayers, indicate that if Ca^{++} is present at a low concentration in one of the bathing solutions then addition and removal of H7 causes, respectively, a reduction and increase of *G* (M.M. Jaeger and Lacaz-Vieira *unpublished results*). In a previous study (Lacaz-Vieira & Kachar, 1996) in frog urinary bladder TJs were opened by apical hypertonicity while a lower free Ca^{++} concentration (0.35 mM) was present in the inner solution. This low basolateral Ca^{++} concentration was sufficient to maintain the TJs closed but not enough to induce TJ recovery when the apical hypertonicity was removed. In this case, it was observed that PKC inhibition with H7 (100 μM) helped junction recovery, that otherwise did not occur at this low Ca^{++} concentration. This response may now be interpreted as indicating that TJ opening or closing results from a delicate balance between opening and closing mechanisms, and that PKC inhibition altered the balance by partially blocking the opening circuit leading to a predominance of the opposite mechanism, as indicated by TJ closing in response to apical hypertonicity removal (Lacaz-Vieira & Kachar, 1996). In consonance with this same interpretation, diC8, a PKC activator, showed an opposite effect when apical hypertonicity was removed, preventing complete TJ recovery in response to a rise of apical Ca^{++} concentration (Lacaz-Vieira & Kachar, 1996).

Another interesting observation was an upregulation of PKC that apparently occurs in response to its inhibition by H7 since an FCSA performed 8 min after H7 removal shows a statistically shorter TJ opening time constant than that in the control situation observed in the same piece of tissue, prior to H7 test (Fig. 3). In apparent consonance with this observation is the down-regulation of PKC induced by the converse maneuver of prolonged activating of PKC by phorbol esters in LLC-PK_{1A} monolayers (Ellis et al., 1992).

The presence of Ca^{++} in the apical medium is not essential for the stability of the TJs, which remain closed in the complete absence of apical Ca^{++}, nor is it essential for junction closing in response to Ca^{++} return to the basolateral solution (Castro et al., 1993; Jovov et al., 1994; Lacaz-Vieira & Kachar 1996; Lacaz-Vieira et al., 1999). However, the presence of apical Ca^{++} may significantly affect the time course of junction opening during an FCSA, depending on its concentration and when apical Ca^{++} is applied. The rate of TJ opening, evaluated by the rate of *G* increase in a FCSA, is reduced (increase of the time constant) when Ca^{++} is present in the apical solution. A high $[Ca^{++}]_{ap}$ may keep the TJs insensitive to a Ca^{++}-free basolateral solution (Lacaz-Vieira, 1997), the TJs remaining closed for long periods of time. Addition of Ca^{++} to the apical solution during the process of TJ opening in an FCSA may affect the time course of *G* increase, the effect depending on $[Ca^{++}]_{ap}$ (Fig. 5*B* and *C*), as compared to a control condition in the absence of apical Ca^{++} (Fig. 5*A*). Normally, a step rise of $[Ca^{++}]_{ap}$ to values of the order of 0.3 mM is sufficient to reduce the

rate of *G* increase. Higher concentrations determine a halt in the process of *G* increase, inducing partial *G* recovery, a process which escapes subsequently, leading to a late increase of *G* towards a new steady-state level. This effect was interpreted as resulting from Ca^{++} entering the permeabilized TJs and interacting with the Ca^{++} sites of the *zonula adhaerens* where it triggers TJ closure, an effect that is not due to Ca^{++} entering the cell through apical membrane Ca^{++} channels (Lacaz-Vieira & Kachar, 1996). However, the possibility of Ca^{++} crossing the open TJ, reaching the intercellular space and from there the cell milieu affecting TJ permeability is a possibility that cannot be discarded, since in A6 cell monolayers cell Ca^{++} appears to modulate TJ resistance (Jovov et al., 1994). The particular experimental condition of having Ca^{++} only in the apical solution and the TJs located midway between the Ca^{++} source (apical solution) and the Ca^{++}-binding sites of *zonula adhaerens*, configures a control negative feedback loop situation. This feedback allows us to understand the escape phase that follows the partial recovery of *G* which is observed in response to a step rise of $[Ca^{++}]_{ap}$ during the course of TJ opening in a FCSA. The sudden rise of $[Ca^{++}]_{ap}$ causes Ca^{++} diffusion through the open TJs, increasing Ca^{++} concentration at the Ca^{++} binding sites of *zonula adhaerens*. This triggers a regulatory TJ closing, characterized by a sharp drop of *G* which in turn limits diffusion of Ca^{++} from the apical solution to the *zonula adhaerens.* The inner compartment, where $[Ca^{++}]_{bl} = 0$ mM, behaves as a Ca^{++} sink allowing Ca^{++} to diffuse from the *zonula adhaerens* region to the inner solution leading to a drop of the Ca^{++} concentration at the Ca^{++} binding sites of *zonula adhaerens*, causing a later rise of *G*, as observed experimentally (Figs. 5*B* and *C*).

Frequently, a rise of $[Ca^{++}]_{ap}$ during TJ opening in an FCSA induces *G* oscillations of low frequency that normally start when Ca^{++} is added to the apical solution (Figs. 5*C* and 6*A*). Oscillations can also be triggered in response to basolateral Ca^{++} removal when Ca^{++} is already present in the apical solution. In this case, oscillations are superimposed to the progressive increase of *G* that results from lowering $[Ca^{++}]_{bl}$ (Fig. 6*B*). Oscillations of tissue electrical conductance most certainly result from oscillations of TJ permeability which, in turn, can be explained by the negative feedback control loop, where the effector, the TJ barrier, limits the access of the control signal (Ca^{++}) to its site of action (the Ca^{++} sites of E-cadherin in the *zonula adhaerens*). Negative feedback loops tend to make an accurate and stable performance since they endeavor to continually reduce any error to an acceptable value. However, under certain conditions, the corrective action can produce unstable operation and the system may drive to limiting values or show oscillations (Weyrick, 1975; Ogata, 1998). Time delays or lags in the response are normally the cause of instability. Inherent time delays may prevent the stopping of the control action in time to avoid overshoot of the controlled variable, which, in turn, may result in a corrective action in the opposite direction. This may cause the feedback to reinforce rather than oppose the input signal, resulting in continuous or damped oscillations. The theory of automatic controls shows that accuracy and stability are mutually incompatible (Weyrick, 1975). Accuracy is improved as loop gain is increased. However, increase in gain also tends to make the system unstable. The study of these oscillations — their amplitude, frequency, occurrence, and response to drugs — might pay off allowing us to probe the control systems that regulate TJ opening and closing. An example is shown in Fig. 7 where inhibition of PKC by H7 causes a prompt stop of *G* oscillations, which then appear when the inhibitor is removed from the inner bathing solution.

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