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Protein Kinase Inhibitors and the Dynamics of Tight Junction Opening and Closing in A6 Cell Monolayers

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Abstract. This study focuses, in A6 cell monolayers, on the role of protein kinases in the dynamics of tight junction (TJ) opening and closing. The early events of TJ dynamics were evaluated by the fast Ca^{++}-switch assay (FCSA), which consisted of 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 evaluation of the effects of drugs and procedures acting upon the mechanism controlling the TJs. The time courses of TJ opening and closing in response to the FCSA followed single-exponential time courses. A rise of apical Ca^{++} (Ca^{++}_{ap}) causes a reduction of TJ opening rate in an FCSA or even a partial recuperation of *G*, an effect that is interpreted as mediated by Ca^{++}_{ap} entering the open TJs. Protein kinase C (PKC) inhibition by H7 at low concentrations caused a reduction of the rate of junction opening in response to Ca^{++}_{bl} removal, without affecting junction closing, indicating that PKC in this preparation is a key element in the control of TJ opening dynamics. H7 at 100 μM completely inhibits TJ opening in response to Ca^{++}_{bl} withdrawal. Subsequent H7 removal caused a prompt inhibition release characterized by a sharp *G* increase, a process that can be halted again by H7 reintroduction into the bathing solution. Differently from the condition in which Ca^{++} is absent from the apical solution, in which H7 halts the process of *G* increase in response to a FCSA, when Ca^{++} is present in the apical solution, addition of H7 during *G* increase in an FCSA not only induces a halt of the *G* increase but causes a marked recuperation of the TJ seal, indicated by a drop of *G*, suggesting a cooperative effect of Ca^{++} and H7 on the TJ sealing process. Staurosporine, another PKC inhibitor, differently from H7, slowed both *G* increase and *G* decrease in an FCSA. Even at high concentrations (400 nM) staurosporine did not completely block the effect of Ca^{++} withdrawal. These discrepancies between H7 and staurosporine might result from distinct PKC isoforms participating in different steps of TJ dynamics, which might be differently affected by these inhibitors. Immunolocalizations of TJ proteins, carried out in conditions similar to the electrophysiological experiments, show a very nice correlation between ZO-1 and claudin-1 localizations and *G* alterations induced by Ca^{++} removal from the basolateral solution, both in the absence and presence of H7.

Key words: Tight junction — Protein kinases — Protein kinase C — Calcium — Paracellular conductance — H7 — Staurosporine — Fast Ca^{++}-switch assay — ZO-1 — Claudin-1

Introduction

Epithelial membranes are polarized structures with cells held together by the junctional complex, the tight junction (TJ) being the most apical component of the junctional complex. TJs form a morphological and functional boundary between the apical and basolateral cell surface domains of epithelia and endothelia, and regulate transport along the paracellular route. (For recent reviews, *see:* Cereijido, Ponce & González-Mariscal, 1989; Balda et al., 1992; Rubin, 1992; Schneeberger & Lynch, 1992; Anderson et al., 1993; Citi, 1993; Anderson & Van Itallie, 1999; Rubin & Staddon, 1999; Balda & Matter, 2000; González-Mariscal, Betanzos & Avila-

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Flores, 2000; Kniesel & Wolburg, 2000). The TJs are dynamic structures which respond to a series of physiological, pathological and pharmacological challenges (Cereijido et al., 1988; Bentzel, Palant, and Fromm, 1991; Schneeberger & Lynch, 1992). A complete picture of the regulatory mechanisms involved in the control of the TJs is far from being fully developed. Depletion of extracellular Ca^{++} induces TJ dissociation both in natural epithelia (Sedar & Forte, 1964; Galli, Camilli & Meldolesi, 1976; Meldolesi et al., 1978; Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997) and in cultured monolayers (Cereijido et al., 1978; Martinez-Palomo et al., 1980; Lacaz-Vieira et al., 1999), and a rise of extracellular Ca^{++} concentration triggers the reassembly and resealing of TJs (González-Mariscal, Chavez de Ramirez & Cereijido, 1985). A delicate mechanism controlling the TJs involves recognition sites, signalling systems and intracellular events that are triggered by Ca^{++} acting on the extracellular aspect of the cells (Balda et al., 1991). The Ca^{++}-dependent adhesion molecule E-cadherin (Gumbiner, 1991; Gumbiner, Stevenson & Grimaldi, 1988; Vestweber & Kemler, 1985) plays a key role in the transduction of the Ca^{++} signal across the plasma membrane. The steps and cell components responsible for the propagation inside the cell of the Ca^{++}-triggered signal are still only partially understood. Based on pharmacological studies it has been suggested that G proteins, phospholipase C, protein kinase C (PKC), and calmodulin might be involved (Balda et al., 1991). TJ biogenesis appears to be regulated, in part, by classical signal transduction pathways involving heterotrimeric G proteins, release of intracellular Ca^{++} and activation of PKC, although many of the details of the signalling pathways have yet to be characterized (Denker & Nigam, 1998). Signalling pathways involved in TJ regulation comprise, in addition, serine, threonine, and tyrosine kinases, extra- and intracellular calcium levels, cAMP levels, proteases, and TNF alpha, common to most of these pathways being the modulation of cytoskeletal elements (Kniesel & Wolburg, 2000). Cell-cell adhesion in TJs does not appear to rely on a single type of intercellular interaction but is mediated by different types of adhesive interactions involving occludin, claudins and the junction-associated membrane protein (JAM) (Balda & Matter, 2000).

The use of different protein kinase activators and inhibitors has revealed that protein phosphorylation plays an important role in TJ assembly and function (Balda et al., 1991; Citi, 1992; Balda et al., 1993; Denisenko et al., 1994; Citi & Denisenko, 1995; Stuart & Nigam, 1995). However, a positive correlation had not been obtained between TJ assembly and phosphorylation level until it was shown that phosphorylation of occludin is a key step in tight junction assembly (Sakakibara et al., 1997). Although it can be said that PKC activation leads to leakiness in epithelial tight junctions (Mullin & McGinn, 1988; Mullin et al., 1990; Winter et al., 1991; Nathanson et al., 1992; Stenson et al., 1993; Burgstahler & Nathanson, 1995) and PKC inhibition has the opposite effect (Nathanson et al., 1992; Stenson et al., 1993; Citi & Denisenko, 1995), these statements cannot be generalized to all epithelial membranes. Thus, phorbol 12-myristate 13-acetate (PMA), an activator of PKC, decreases the tight junction conductance during the reorganization of LLC-PK1A monolayers, but has the opposite effect in LLC-PK1B4, MDCK, and MDCK4 cells, indicating that PKC regulates the development of the occluding junctions, but through different mechanisms depending on the characteristics of the cells (Ellis et al., 1992). Another contrasting example is the effect of 1,2-dioctanoylglycerol (diC8), an activator of PKC, promoting in MDCK cells the assembly of TJ in low extracellular Ca^{++} with a decrease of the permeability of the intercellular space (Balda et al., 1993). PKC requirement for the proper assembly of TJs is shown by the inhibitor of PKC, calphostin C, that markedly inhibited development of transepithelial electrical resistance and causes a delay in the sorting of the TJ protein ZO-1 to the tight junction (Stuart & Nigam, 1995). Among different PKC isoforms the alpha (PKCα) (Rosson et al., 1997) and the Ca^{++}-independent delta-isoform of protein kinase C (PKCδ) (Mullin et al., 1998) are known to contribute to TJ organization in epithelia. Among the potential candidates as targets of PKC are the transmembrane TJ proteins and the associated cytosolic TJ proteins. During junction assembly by calcium-switch, H7 did not change the specific phosphorylation of the immunoprecipitated cingulin, however, it prevented the increase in the amount of cingulin in the immunoprecipitates, suggesting that H7 may block TJ assembly by interfering with cellular processes that lead to the accumulation and stabilization of TJ proteins at sites of cell-cell contact (Citi & Denisenko, 1995). Vinculin phosphorylation by PKC has been suggested to be a crucial step in the correct assembly of the epithelial junctional complex (Perez-Moreno et al., 1998). It has been hypothesized that PKC activation alters transepithelial conductance via the perijunctional actomyosin ring (PAMR) by changing the phosphorylation of myosin light chain (MLC). The decreased phosphorylation of MLC likely reduces PAMR tension, leading to decreased TJ permeability (Turner et al., 1999). In addition it was shown that antibodies to E-cadherin block junction formation induced by extracellular Ca^{++} and diC8 counteracts this effect, suggesting that PKC may be in the signalling route activated by E-cadherin-mediated cell-cell adhesion (Balda et al., 1993).

This study focuses, in A6 cell monolayers, on the effects of protein kinase inhibitors H7 and staurosporine on the kinetics of TJ opening and closing in a FCSA,

as well as on the immunolocalizations of ZO-1 and claudin-1.

Materials and Methods

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, 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 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.

Electrical Measurements

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. 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. Unstirred layers on the surfaces of the monolayer were minimized by directing the incoming fluid towards the monolayer. Each compartment was drained through a spillway open to the atmosphere, so that the pressure inside each compartment was kept 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.

A conventional voltage-clamp (DVC-1000, World Precision Instruments) with continuous feedback was used. Saturated calomel half-cells with 3 M KCl agar bridges were used to monitor the electrical potential difference across the monolayer. Current was passed through Ag-AgCl electrodes and 3 M KCl agar bridges to give a uniform current density across the monolayer. The monolayers were kept clamped at a sinusoidal voltage of ±5 mV peak to peak and 0.1 Hz throughout the experiment. The clamping current was continuously recorded with a strip-chart recorder and also digitized through an analog-to-digital converter at a frequency of 1 kHz (Digidata 1200 and Axotape 2.0, Axon Instruments) and recorded in a computer for further processing.

Solutions and Drugs

The composition of the solutions used to bathe the inner side of the monolayers were (in mM): NaCl-Ringer: NaCl 115, $KHCO_3$ 2.5, $CaCl_2$ 1.0; Ca^{++}-free NaCl-Ringer: NaCl 115, $KHCO_3$ 2.5. These Ringer solutions had pH 8.2 after aeration. The apical bathing fluid was a simple salt solutions of KCl 75 mM, non-buffered, or buffered with 10 mM HEPES, pH adjusted with KOH. H7 (1-5(isoquinolinesulfonyl)-2-methyl-piperazine) and all salts were purchased from Sigma-Aldrich Co., and staurosporine from Molecular Probes.

Fast Ca^{++}-Switch Assay

The junctions were opened by removal of basolateral Ca^{++}. Subsequent resealing of the TJs was induced by the reintroduction of Ca^{++} in the basolateral fluid. The action of drugs on the TJs was tested by introducing them to the apical or basolateral solutions and observing the effects on the dynamics of TJ opening and closing or on immunolocalization of ZO-1 and claudin-1. 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 FCSA due to the buffering effect on the Ca^{++} concentration in this compartment. Changes in TJ permeability were gauged through changes of transepithelial electrical conductance (G) determined in the absence of apical Na^+, a condition in which G is a reliable measure of TJ permeability (Lacaz-Vieira & Kachar, 1996).

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).

Immunolocalization of TJ Proteins

We followed the distribution of two tight junction proteins, one cytoplasmic (ZO-1) and one transmembrane (claudin-1), during the FCSA in four situations:

1. Before Ca^{++}_{bl} withdrawal (control monolayers);
2. After Ca^{++}_{bl} withdrawal;
3. After Ca^{++}_{bl} withdrawal in the presence of the PKC inhibitor (H7)
4. After Ca^{++}_{bl} withdrawal in the presence of H7 in monolayers pretreated for 5 min with this PKC inhibitor.

After each experiment was carried out, the cell monolayers were fixed with 1% paraformaldehyde in phosphate buffered saline solution (PBS), pH 7.4, for 10 min at room temperature (RT). After fixation, the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min at RT. The antibodies used were: mouse anti-ZO-1 (stock solution: 0.5 mg/ml in PBS, pH 7.4, containing 0.1% sodium azide) and rabbit anti-claudin-1 (stock solution: 0.5 mg/ml in PBS, pH 7.4, containing 0.05% sodium azide). Both antibodies were purchased from Zymed Lab (San Francisco, CA) and diluted 1:50 in PBS. The reactions were visualized with fluorescein-conjugated donkey anti-mouse (for ZO-1) and anti-rabbit (for claudin-1). All incubations were done for 45 min at RT. After labeling, the filters were excised from their supports and sandwiched between a glass slide and a coverslip in a mounting medium (Prolong™ antifade kit P-7481; Molecular Probes, Eugene, OR).

The observations and photographic recording were carried out in a Zeiss Axiophot 2 fluorescence microscope (Carl Zeiss Oberkochen, Germany) using an objective 63X Plan Neofluor 1.4 N.A.

Abbreviations and Conventions

I: Clamping current, in $\mu A\ cm^{-2}$. Positive current corresponds to the transport of positive charges across the monolayer from the apical to the basolateral solution. *V:* Electrical potential difference across the monolayer, 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

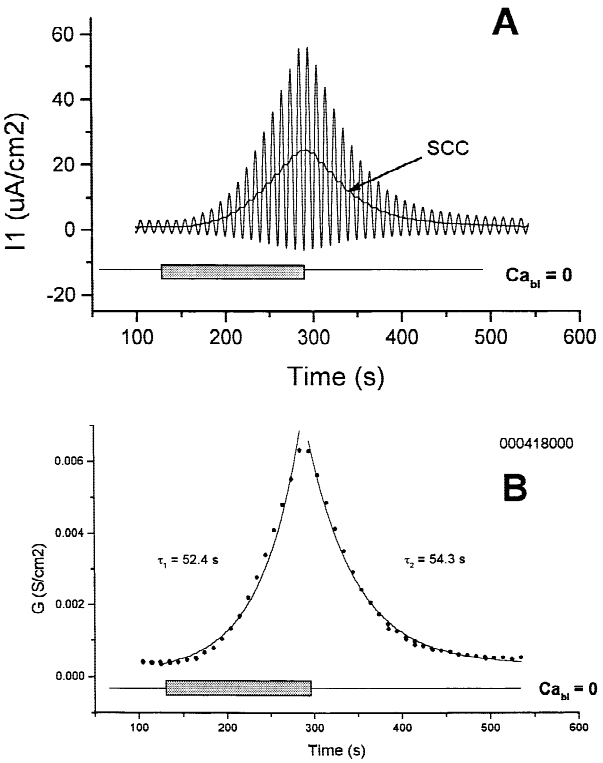

Fig. 1. Representative experiment of tight junction (TJ) opening and closing, evaluated by changes in monolayer electrical conductance (*G*) in response to a fast Ca^{++}-switch assay (FCSA) (*see* Materials and Methods). The apical solution, when not otherwise indicated, was 75 mM KCl, and the basolateral, NaCl-Ringer's. The grey rectangles indicate the period in which there were changes in concentration, which are then specified at the right end of the lines passing through the rectangles. When units are not specified, the concentrations are in mM. (*A*) Plot of the clamping current (as a function of time) modulated by a sinewave voltage of 0.1 Hz and 5 mV peak to peak (*see* Materials and Methods). The short-circuit current (SCC) was evaluated by a fast Fourier transform filter (200 points averaging) applied to the clamping current. (*B*) Time course of *G* in response to FCSA. As can be seen, the increase and decrease of *G* follow single exponential time courses, characterized by single time constants, τ_1 and τ_2, respectively.

graphing software Origin™ (version 5) (Microcal Sofware). 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. $Ca^{++}{}_{ap}$ and $Ca^{++}{}_{bl}$: Apical and basolateral Ca^{++}, respectively. TJ: tight junction; FCSA: fast Ca^{++}-switch assay; PKC: protein kinase C.

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 early kinetics events of TJ opening and closing in A6 cell monolayers. 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 later compared to conditions in the presence of one of the PKC inhibitors used, H7 or staurosporine) were carried out to characterize the monolayers' electrical conductance (*G*) response to a fast Ca^{++}-switch assay (FCSA), which consisted of 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 & Kachar, 1996; Lacaz-Vieira et al., 1999; Lacaz-Vieira, 2000). In the control condition, monolayers were bathed on the apical side by a nominally Ca^{++}-free 75 mM KCl solution, to demote the contribution of the transcellular Na^+ pathway to the overall tissue electrical conductance, and on the basolateral side by NaCl Ringer's solution. Monolayers were previously equilibrated for several minutes in these solutions, showing a stable *G* of $4.21 \times 10^{-4} \pm 3.3 \times 10^{-5}$ S/cm^2 ($n = 37$). After an average delay of 97 ± 12.7 sec ($n = 37$), *G* begins to increase at a fast growing rate in response to basolateral Ca^{++} removal. If a Ca^{++}-free basolateral solution is kept for several minutes, *G* increases markedly, eventually reaching a plateau level. When this high steady-state conductance is reached, return of Ca^{++} to the basolateral medium induces the recovery process that is very slow compared to the time course of *G* increase in response to Ca^{++} removal. In some cases, full *G* recovery was never observed in a reasonable time. To avoid this situation and to standardize the experimental protocol in order to work in a condition in which reversible TJ opening and closing were obtained, minor perturbations of the TJ seal were induced. For this, the process of junction opening was always halted when *G* reached values of the order of 6×10^{-3} S/cm^2, by returning Ca^{++} to the basolateral solution. This led to a complete recovery of the TJ seal in all cases, as indicated by the return of *G* to initial values. In this particular condition, the time courses of *G* increase and decrease in response to FCSA followed single-exponential time courses, which could be characterized by single time constants (Fig. 1). 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 59.7 ± 7.2 sec ($n = 12$). The decrease of *G* in response to Ca^{++}-return to the basolateral solution followed a first-order exponential decay, according to: $G = G_o + A_2 \exp-((t - t_o)/\tau_2)$, 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 61.0 ± 9.4 sec ($n = 12$). Figs. 1*A* and 1*B* show, respectively, for a representative control experiment, the clamping-

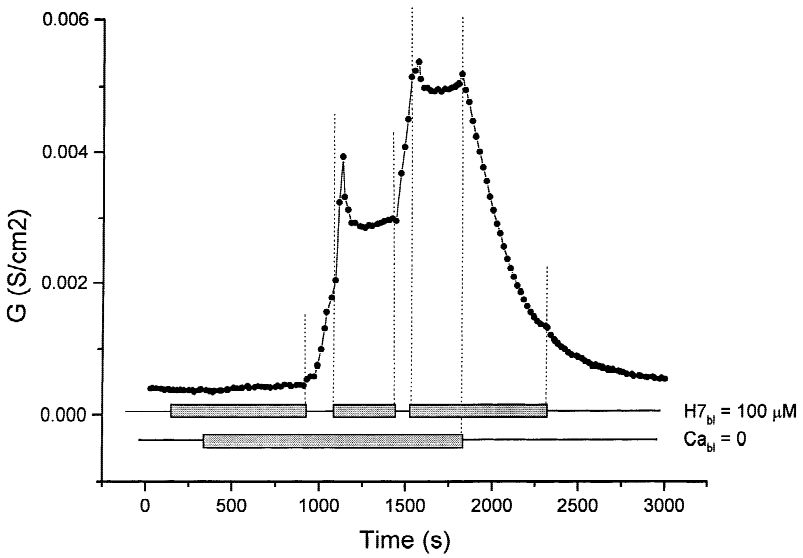

Fig. 2. Representative experiments (from a group of 6 experiments) on the effect of protein kinase inhibitor H7 at 100 μM concentration on the time course of *G* increase and decrease in response to FCSA. The apical solution was 75 mM KCl, and the basolateral, NaCl-Ringer's. The monolayer was exposed to the inhibitor for 4 to 5 min before removing Ca^{++} from the basolateral solution. When basolateral Ca^{++} was removed, no increase of *G* was observed, in contrast to what occurs in the control condition in the absence of H7. A subsequent removal of the inhibitor almost immediately triggers junction opening that again is blocked by H7. Finally, it can be seen that H7 removal does not affect the time course of *G* recovery induced by the return of basolateral Ca^{++}.

current response to FCSA and the exponential fitting to both the increase and decrease of G.

For the experiments in which the PKC inhibitor H7 was used, each assay started with an initial control FCSA carried out to determine the untreated-tissue response pattern. One experimental group ($n = 8$) was carried out to evaluate tissue sensitivity to the PKC inhibitor, H7, at different concentrations (10 to 100 μM), added to the inner solution. A period of equilibration elapsed, and subsequent FCSAs were carried out at successive time intervals. The effect of H7 became noticeable around 10 μM and reached full effect at 100 μM. In a second group of experiments, H7 was used at its full-effect concentration (100 μM) to evaluate its effect on the time course of TJ opening and closing. The inhibitor was added to the inner solution and after 5 min, basolateral Ca^{++} was removed and no increase of *G* was observed for 8 to 10 min, as would be expected from the previous group (Fig. 2 is a representative experiment). Then, H7 was removed from the inner solution, triggering an almost immediate onset of *G* increase, which could be halted in a few seconds upon H7 reintroduction, which was followed by a small but consistent recovery of *G*. A sequence of inhibition release and block of *G* increase could again be induced upon H7 removal and addition, respectively. Finally, Ca^{++} return to the basolateral medium caused a complete *G* recovery, which was not affected by the presence of the inhibitor, as indicated by the fact that H7 removal during *G* recovery did not affect the time course of *G* decrease. The experiments with H7 indicate that PKC plays an important role in TJ opening without appreciably affecting the reverse process of junction recovery in response to Ca^{++}-return to the basolateral solution. A group of experiments ($n = 5$) was carried out to compare the effects of simultaneously removing Ca^{++} from the basolateral solution and adding H7 to the same solution (Fig. 3 depicts a representative experiment). In all cases, the effect of Ca^{++} removal (characterized by an increase of *G*) was seen, indicating that the diffusion delay for H7 reaching its site of action is larger than that for Ca^{++} reaching its binding sites. However, the time difference between the moment *G* starts to increase in response to Ca^{++} withdrawal and the blockade induced by H7 is quite small, averaging 38 ± 5 sec ($n = 5$).

Another PKC inhibitor, staurosporine, was tested on the FCSA response, and a representative experiment of a group of 6 is shown in Fig. 4. As can be seen, staurosporine (400 nM) caused a marked slowing of both, *G* increase in response to basolateral Ca^{++} removal (increase of the time constant, τ_1), and *G* decrease in response to Ca^{++} return to the basolateral solution (increase of the time constant, τ_2), the effect on *G* decrease being more intense, as evaluated by the effect on the time constants. Mean values of τ_1 and τ_2 for this experimental group are the following: (a) Control condition before staurosporine: $\tau_1 = 54.7 \pm 7.2$ sec and $\tau_2 = 58.1 \pm 9.3$ sec. (b) 30 min after adding staurosporine (400 nM) to the inner solution: $\tau_1 = 69.9 \pm 9.2$ sec and $\tau_2 = 107.7 \pm 15.2$ sec. (c) 60 min after adding staurosporine (400 nM) to the inner solution: $\tau_1 = 171.0 \pm 20.1$ sec and $\tau_2 = 319.8 \pm 39.5$ sec. The effect of staurosporine, differently from that of H7, which takes place rapidly in response to drug addition or removal, builds up slowly and normally takes almost one hour to attain a stable effect. Higher concentrations of staurosporine tested never caused a complete block of junction dynamics in response to FCSA, as it was observed with H7.

Effect of Apical Ca^{++} on the FCSA

In the course of junction opening in response to basolateral Ca^{++} removal, addition of Ca^{++} to the apical solution

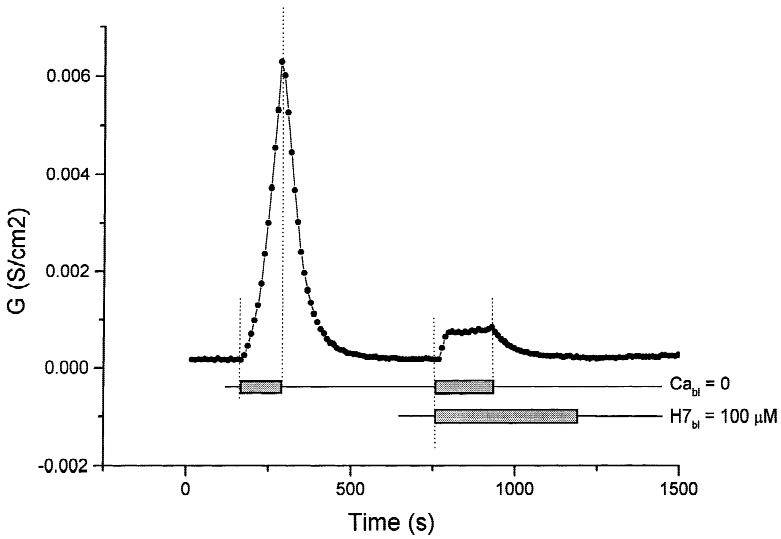

Fig. 3. Representative experiments (from a group of 4 experiments) on the effect of protein kinase inhibitor H7 at 100 μM concentration on the time course of *G* increase and decrease in response to a FCSA. The apical solution was 75 mM KCl, and the basolateral, NaCl-Ringer's. The monolayer was initially submitted to a control FCSA. Then, when *G* had returned to a value similar to the initial steady-state level, Ca^{++} was removed from and simultaneously H7 was added to the basolateral solution. As can be observed, *G* starts to increase in response to basolateral Ca^{++} removal and after a few seconds this process is completely halted. Finally, when Ca^{++} is reintroduced in the basolateral solution, *G* recovery takes place.

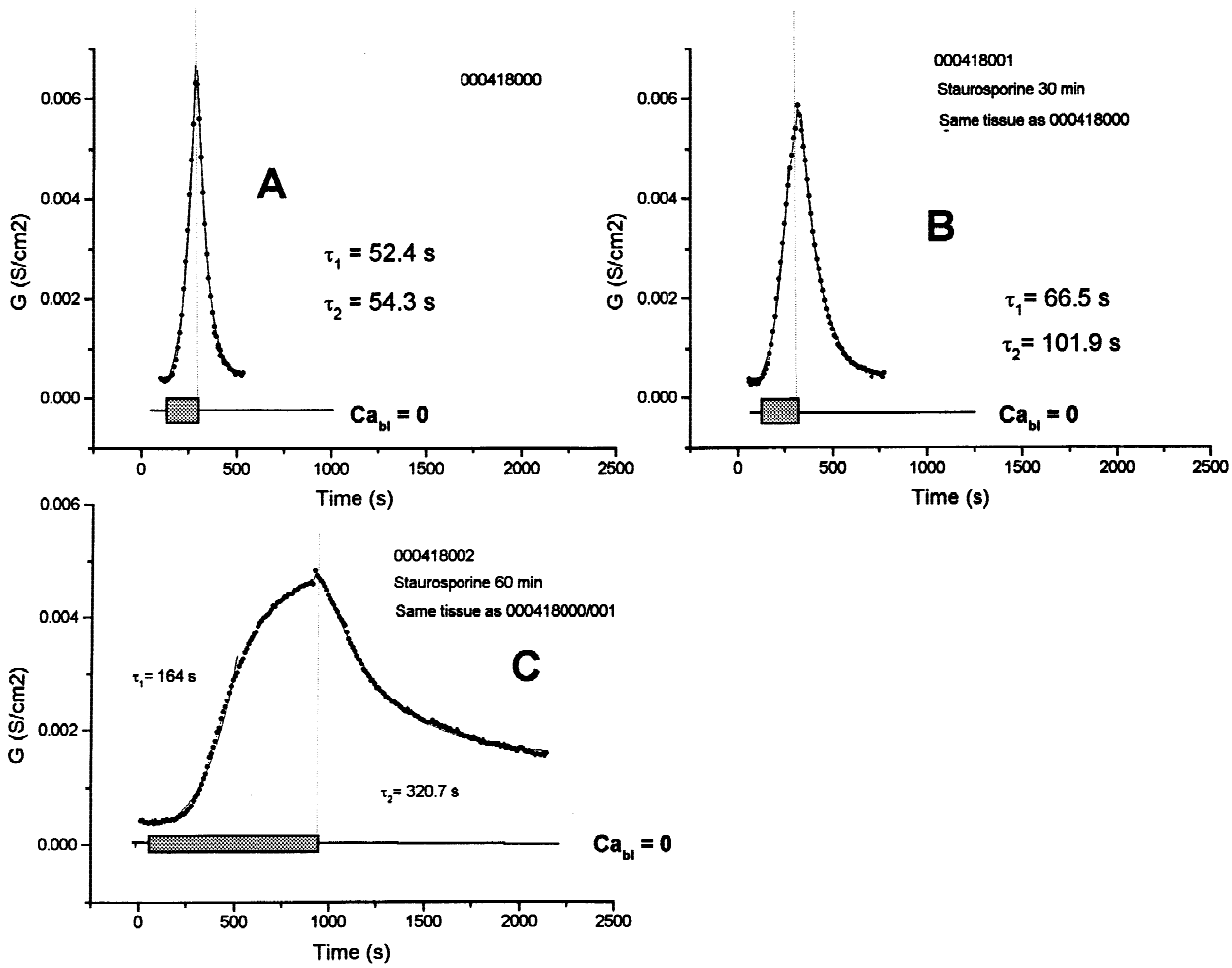

Fig. 4. Representative experiment (from a group of 6) of the effect of staurosporine (400 nM) added to the inner solution on monolayer electrical-conductance (*G*) responses to a fast Ca^{++}-switch assay (FCSA). The apical solution was 75 mM KCl, and the basolateral, NaCl-Ringer's. (*A*) Control response in the absence of staurosporine. (*B*) and (*C*) Responses to FCSA elicited 30 min and 60 min, respectively, after tissue exposure to staurosporine.

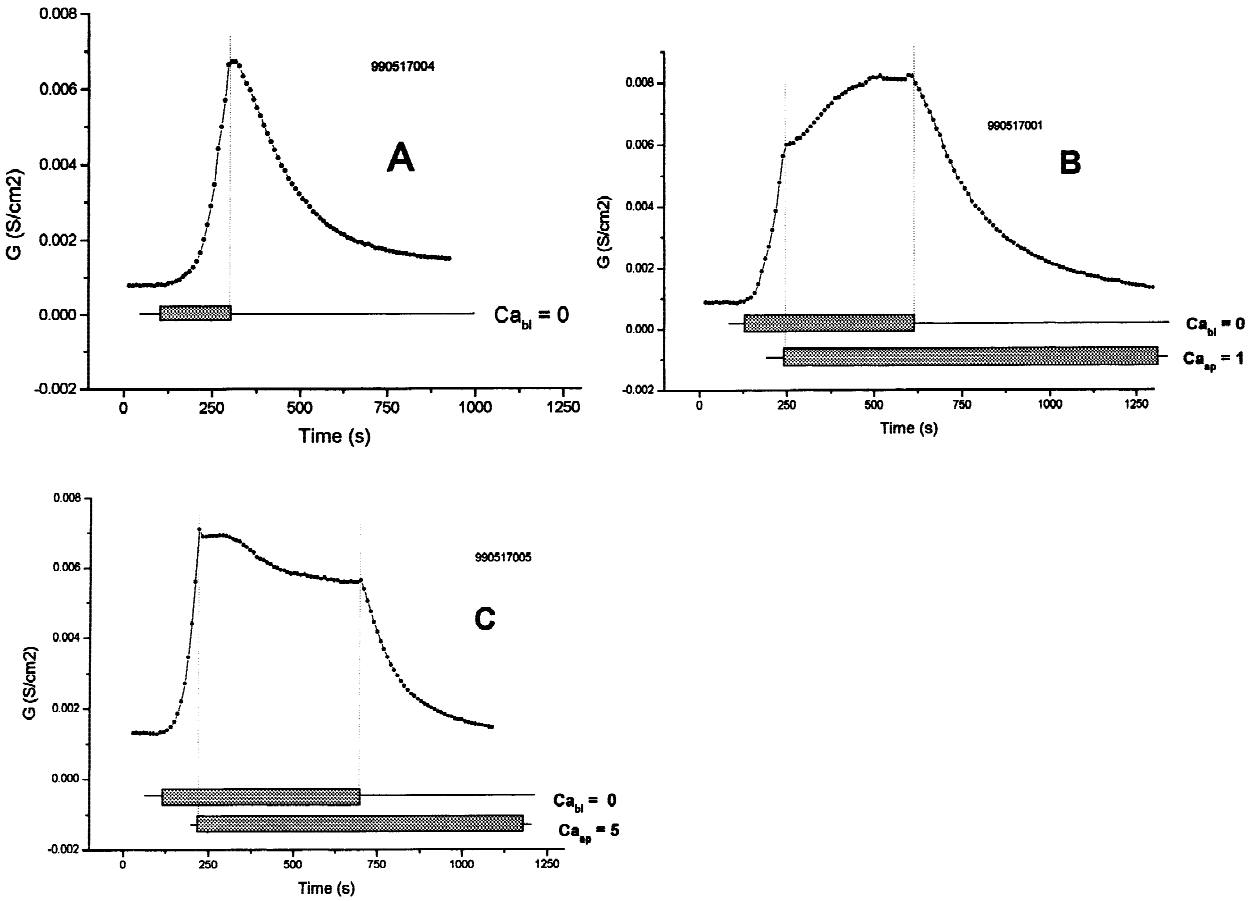

Fig. 5. Representative experiments on the effect of apical Ca^{++} on monolayer electrical-conductance (*G*) responses to a fast Ca^{++}-switch assay (FCSA). The apical solution was 75 mM KCl, and the basolateral, NaCl-Ringer's. (*A*) Control responses to FCSA carried out in the absence of apical Ca^{++}. The effects of rising apical Ca^{++} concentration during the process of *G* increase in response to basolateral Ca^{++} removal are shown in *B* (apical Ca^{++} = 1 mM) and *C* (apical Ca^{++} = 5 mM).

has an almost instantaneous effect on TJ dynamics. At low concentrations, apical Ca^{++} retards the process of *G* increase in response to basolateral Ca^{++} removal. Higher apical Ca^{++} concentrations may halt the process of *G* increase and causes partial *G* recovery, with *G* evolving to a steady-state condition as shown in Fig. 5 for a representative case. These results indicate that apical Ca^{++} is able to reach the Ca^{++} sites that control TJ dynamics. In a previous study in frog urinary bladders we have shown that the effect of apical Ca^{++} most certainly results from Ca^{++} ions entering the open TJs and interacting with E-cadherin molecules present at the zonula adhaerens, and was not a consequence of a rise of cytosolic Ca^{++} concentration due to Ca^{++} flux through apical Ca^{++} channels (Lacaz-Vieira & Kachar, 1996). The stationary level of *G* attained in response to apical Ca^{++} is an interesting condition to work with since the TJs are kept in an unstable condition midway between totally closed and fully leaky junctions, being sensitive to small perturbations of their control parameters. This condition was used to test the effect of H7, and Fig. 6 depicts a representative experiment of a group of 5. As can be seen, PKC inhibition with H7 (100 μM) during the stationary condition attained in response to apical Ca^{++} causes a very pronounced recovery of the TJ seal as indicated by the much larger drop of *G* than that observed when Ca^{++}-free bathing solutions were used (Fig. 2), suggesting that PKC inhibition by itself is able to stop *G* increase, but an appreciable recovery of *G* might need the presence of a small extracellular Ca^{++} concentration acting on the Ca^{++} sites that control the TJs.

ZO-1 and Claudin-1 Immunolocalization

These experiments were carried out aiming to compare the electrophysiological findings with the structural alterations of TJ associated proteins in FCSA. Two TJ-associated proteins, ZO-1, a cytoplasmic protein (Stevenson et al., 1986), and claudin-1, a transmembrane

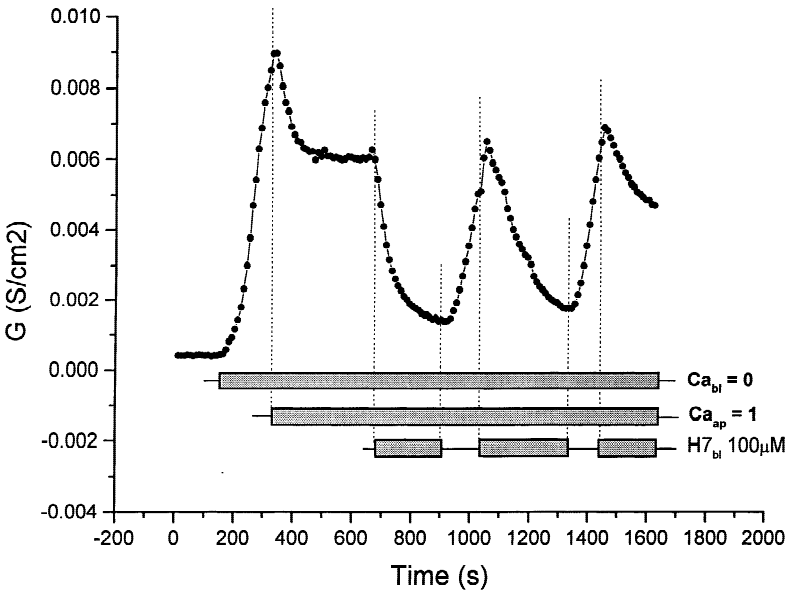

Fig. 6. Representative experiments (from a group of 5 experiments) on the effect of protein kinase inhibitor H7 at 100 μM concentration on *G* steady-state level attained after apical Ca^{++} was added during the rising phase of *G* in FCSA. The apical solution was 75 mM KCl, and the basolateral, NaCl-Ringer's. Sequential additions and removals of H7 caused marked decreases and increases of *G*, which occur with minor delays after solution changes.

protein (Furuse et al., 1998) were studied. The results are summarized in Fig. 7. In all cases the culture medium was removed and the monolayers rinsed in NaCl-Ringer's solution on both sides. Then, the same Ringer's solution was kept on the basolateral side and the apical bathing medium was replaced by a 75 mM KCl solution. Panels *A* and *B* are control experiments in which the monolayers were fixed (*see* Methods) without any further treatment. In these control preparations the cells in the monolayer are perfectly confluent and ZO-1 appears as continuous lines on the cell borders (*A*). Claudin-1 presents a distribution similar to that of ZO-1 at the cell borders, but is also expressed throughout the cell membrane as dots (*B*). Panels *C* and *D* are from experiments in which the basolateral solution was replaced by a Ca^{++}-free NaCl-Ringer's solution and the monolayers fixed after 3 min. As can be seen, after Ca^{++} withdrawal some cells lose, at focal points, cell-cell contact and their polygonal shape, retracting and leading to discontinuities or holes in the monolayer. When Ca^{++}_{bl} is removed, the cell-cell as well as cell-substrate contacts are markedly weakened and do not resist the stress of cell monolayer handling during the immunofluorescence manipulations. These holes, we believe, result from cell separation due to weakness of these contacts. We did not show in the present paper a figure for the complete recovery of the cell monolayer after returning Ca^{++} to the basolateral solution, since this has been previously reported in immunofluorescence images of ZO-1 in A6 cell monolayers submitted to the same protocol, in a paper from our laboratory (Lacaz-Vieira et al., 1999). ZO-1 (*C*) and claudin-1 (*D*) are still present at the cell borders, even in cells with almost no cell-cell contact. This indicates that in these short-term experiments, Ca^{++} removal clearly perturbs cell-cell contact, but the time in Ca^{++}-free medium is not sufficient to allow redistribution of ZO-1 and claudin-1. Panels *E* and *F* are experiments in which the basolateral solution was replaced by a Ca^{++}-free NaCl-Ringer's solution containing H7 (100 μM) and the monolayers were fixed after 3 min. After simultaneous Ca^{++} withdrawal and addition of H7 (100 μM) to the basolateral solution, the cell morphology as well as the distribution of ZO-1 (*E*) and claudin-1 (*F*) remain unchanged, similar to control cell monolayers, except for a few clefts (between arrows) between cells, which could more easily be identified in the ZO-1 preparations (*E*). Panels *G* and *H* are experiments in which the basolateral solution was replaced by a NaCl-Ringer's solution containing H7 (100 μM). After 5 min the basolateral solution was replaced by a Ca^{++}-free NaCl-Ringer's solution containing H7 (100 μM), and the monolayers were fixed after 3 min. The cells are perfectly confluent, indistinguishable from those of the control cell monolayers (*A*, *B*).

Discussion

The present study focuses, in mature A6 cell monolayers, on the role of extracellular Ca^{++} and PKC in the early events of TJ opening and closing, which were assessed by the fast Ca^{++}-switch assay (FCSA) (*see* Methods) (Lacaz-Vieira & Kachar, 1996; Lacaz-Vieira, 1997; Lacaz-Vieira et al., 1999; Lacaz-Vieira, 2000). The FCSA allows assessment of the early kinetic events of TJ opening and closing and evaluation of the 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 can be evoked without

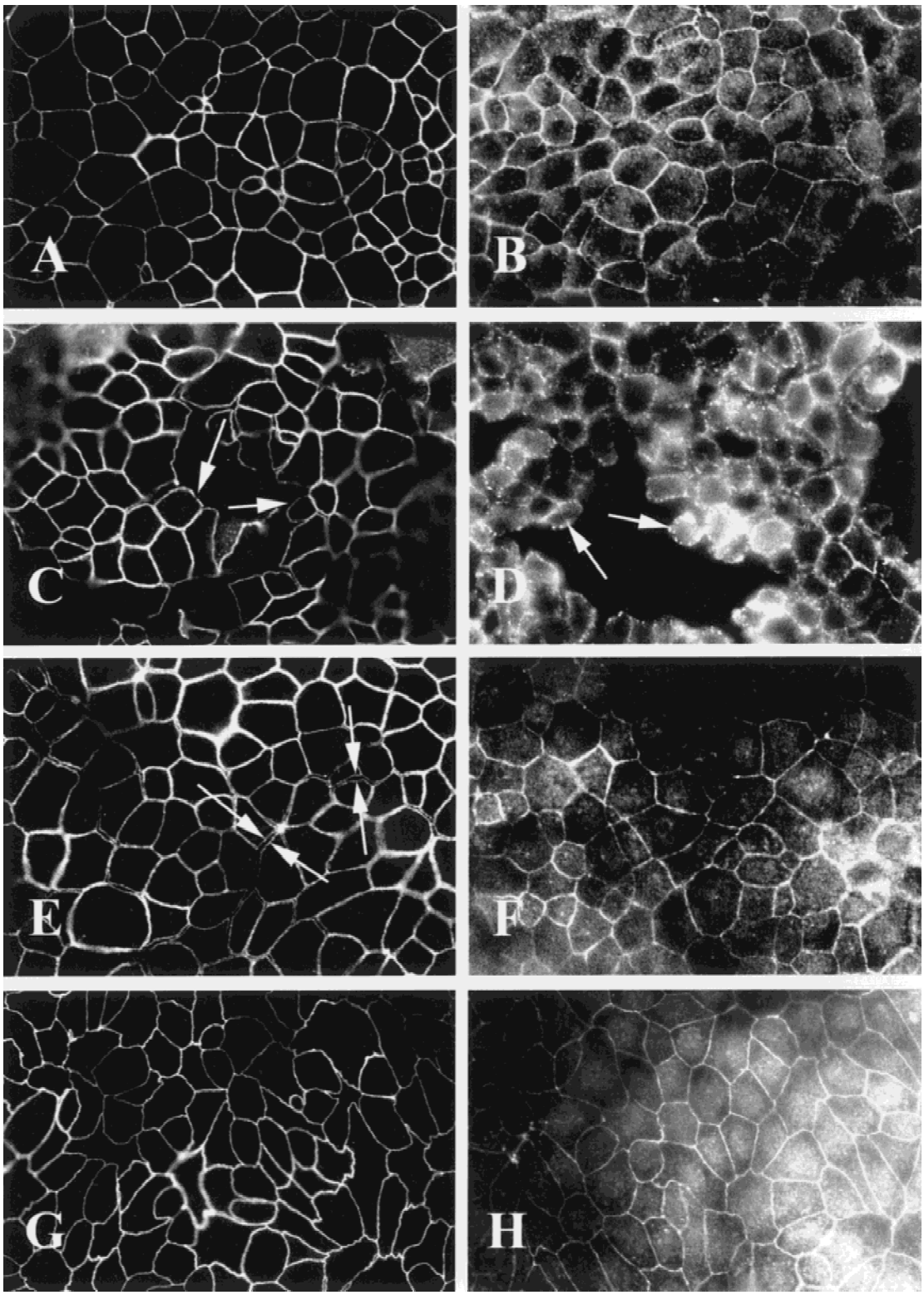

Fig. 7. Immunofluorescence localization of ZO-1 (*A, C, E, G*) and claudin-1 (*B, D, F, H*) in A6 cells following the fast Ca^{++}-switch assay in either the presence or absence of the PKC inhibitor, H7. (*A, B*) Before Ca^{++}_{bl} withdrawal (control preparations) the cells in the monolayer are perfectly confluent and ZO-1 appears as continuous lines on the cell borders (*A*). Claudin-1 presents a distribution similar to that of ZO-1 at the cell borders, but this molecule is also expressed throughout the cell membrane as dots (*B*). (*C, D*) After Ca^{++}_{bl} withdrawal, at focal points, some cells retract, losing cell-cell contact and their polygonal shape, leading to discontinuities or holes in the monolayer. ZO-1 (*C*) and claudin-1 (*D*) are still present at the cell borders, even in cells with no cell-cell contact. (*E, F*) After simultaneous Ca^{++}_{bl} withdrawal and addition of H7 (100 μM) to the basolateral solution, the morphology of the cells as well as the distribution of ZO-1 (*E*) and claudin-1 (*F*) remain unchanged and similar to those of control cell monolayers, except for a few clefts between cells that were more easily identified in the ZO-1 preparations (*E,* between arrows). (*G, H*) After Ca^{++}_{bl} withdrawal in A6 cell monolayers pretreated for 5 min with H7 (100 μM) on the basolateral side, cells labelled for ZO-1 (*G*) and claudin-1 (*H*) are perfectly confluent, indistinguishable from the control cell monolayers (*A, B*).

any appreciable changes in tissue electrical properties, indicating that the challenges induced by the FCSA were completely reversible. It was found that: (1) Tight junction opening- and closing-responses to FCSA follow single-exponential time courses (Fig. 1), characterized by the time constants of *G* increase and decrease, τ_1 and τ_2, respectively. This is in consonance with observations in the CaSki human cervical cell line where time courses also conformed with simple exponential trends (Gorodeski, Jin & Hopfer, 1997), and in frog urinary bladder (Lacaz-Vieira, 2000). (2) Basolateral Ca^{++} removal causes an increase of *G*, which reverts completely upon Ca^{++} return to the basolateral solution. (3) The protein kinase inhibitors, H7 and staurosporine, affect, but differently, the dynamics of TJ opening and closing in FCSA. (4) H7 markedly inhibits TJ opening with negligible effect on the reverse process of TJ closing. (5) At 100 μM, H7 almost immediately blocks TJ opening in FCSA, inducing a small partial recovery, which is magnified when Ca^{++} is present in the apical compartment as compared to the condition of Ca^{++} being absent from the bathing solutions. (6) Staurosporine, in contrast to H7, reduces both TJ opening and closing processes in a FCSA, but in no case was it able to completely block TJ opening or induce *G* recovery.

These observations are in harmony with the knowledge that PKC plays a major role in the dynamics of TJs and that PKC inhibitors are expected to affect these processes. However, an additional contribution of cAMP-dependent protein kinase (PKA) inhibition by H7 cannot be ruled out since in other preparations inhibition of PKA also affects the disruption of TJs induced by low Ca^{++} (Nilsson, Fagman & Ericson, 1996; Klingler et al., 2000). Despite the fact that H7 and staurosporine markedly slow the time course of TJ opening in response to basolateral Ca^{++} removal, differences in their actions on TJ dynamics were observed. The most significant was that staurosporine, in addition to slowing the time course of TJ opening in response to basolateral Ca^{++} withdrawal, also retarded the TJ recovery process induced by the return of Ca^{++} to the basolateral medium. PKC is not just a single molecular species, but a family of kinases comprehending at least 12 known isoforms, some being Ca^{++}-dependent kinases and other Ca^{++}-independent ones (Buchner, 1995; Dekker & Parker, 1994). Of these different PKC isoforms, so far only PKCα and PKCζ have been reported to colocalize with ZO-1 in the region of the TJs (Dong, Stevens & Jaken; 1993; Dodane & Kachar, 1996). In a previous study in the frog urinary bladder, it was shown that the PKC inhibitor H7 slowed down the rate of TJ opening in FCSA without affecting the reverse process of TJ recovery in response to Ca^{++} return (Lacaz-Vieira, 2000). In addition, it was shown that H7 removal causes not only a fast inhibition recovery but also caused an overshoot characterized by a faster rate of *G* increase in response to basolateral Ca^{++} removal, the observed rate rising above the value measured previously to the use of H7, which was interpreted as reflecting an upregulation of PKC activity (Lacaz-Vieira, 2000). This overshoot, clearly observed in frog urinary bladders, was never seen in A6 cell monolayers in similar conditions, indicating differences among tissues in response to PKC inhibition by H7. It is possible that due to different sensitivities of the kinase isoforms to H7 and staurosporine and to their relative importance in the control of junction opening and closing, the effect of these inhibitors on TJ dynamics may differ as described. A clear picture of how PKC controls TJ dynamics is far from being completely understood. Contradictory effects of PKC on TJ dynamics have been reported in MDCK cell monolayers where inhibitors of PKC have been shown to prevent TJ disassembly (induced by lowering extracellular Ca^{++} concentration (Citi, 1992) or after treatment with cytochalasin D (Citi et al., 1994)) or block TJ reassembly in response to a rise of extracellular Ca^{++} concentration (Balda et al., 1991; Nigam et al., 1991). Likely candidates for PKC phosphorylation in response to a Ca^{++}-switch, the TJ-associated proteins, ZO-1, ZO-2, ZO-3 and cingulin, presented no change in their phosphorylation status during a Ca^{++}-switch in the presence of PKC agonists and inhibitors (Balda et al., 1993; Citi & Denisenko, 1995). Conversely, vinculin was found to be phosphorylated during junctional sealing induced by Ca^{++}, suggesting that its phosphorylation may be a crucial step in the correct assembly of the epithelial junctional complex (Perez-Moreno et al., 1998). The fact that H7 practically has no effect on the Ca^{++}-induced recuperation of *G*, while staurosporine markedly slows the time course of *G* recuperation, might be an indication that these two PKC inhibitors affect different steps of the recovery process, possibly acting on different PKC isoforms.

As observed in frog urinary bladders (Lacaz-Vieira, 2000), the presence of apical Ca^{++} affects the dynamics of TJ opening and closing, effects that were interpreted as resulting from Ca^{++} entering open TJs and interacting with the Ca^{++} binding sites of zonula adhaerens (Lacaz-Vieira, 2000), which control the sealing of TJs (Gumbiner et al., 1988). An interesting interaction between apical Ca^{++} and H7 is the fact that addition of H7 (100 μM) to the basolateral solution halts the process of TJ opening in response to basolateral Ca^{++} removal, but when Ca^{++} is present in the apical solution (in a concentration that does not per se induce TJ recovery) H7 causes a marked recovery of the TJ seal. These observations indicate a cooperative effect of low Ca^{++} concentrations at zonula adhaerens and PKC inhibition by H7 on the TJ sealing process.

Immunolocalizations carried out in conditions similar to the electrophysiological experiments showed a

very nice correlation between ZO-1 and claudin-1 localization and *G* alteration induced by Ca^{++} removal from the basolateral solution. Thus, H7-(100 μM) treated monolayers, when submitted to Ca^{++} removal from the basolateral solution, do not show any change of the overall tissue electrical conductance *G* nor of overall cell morphology, as evidenced by ZO-1 and claudin-1 localizations. In contrast, tissues not exposed to H7 when submitted to basolateral Ca^{++} removal, show a marked increase of *G* as well as alterations of cell morphology, which are quite compatible with the increase of tissue electrical conductance induced by basolateral Ca^{++} removal. Particularly, it is worth noting the interesting change in the distribution of claudin-1 from a continuous to a punctate pattern (Fig. 7*D*), while ZO-1 retains the same pattern (Fig. 7*C*) observed in the control condition. This behavior of ZO-1 is similar to that of occludin observed in MDCK cell monolayers in response to a transient Ca^{++} withdrawal (Farshori & Kashar, 1999). In addition, a coherent behaviour was observed when basolateral Ca^{++} removal and H7 addition to the basolateral bathing solution were carried out simultaneously. In this case, the initial increase of *G* followed by a halt in this process is in clear consonance with the small separation of the epithelial cells, which, however, does not progress despite the absence of basolateral Ca^{++}, different from the observed behavior of monolayers submitted to Ca^{++} removal in the absence of H7, where a marked cell separation took place accompanied by cell detachment. The immunolocalization of these two TJ proteins showed changes that occur to cell morphology in response to Ca^{++} withdrawal in either the presence or absence of the PKC inhibitor H7. However, it is important to observe that both proteins (ZO-1 and claudin-1) were localized at the cell borders in all situations, indicating that in FCSA there is not enough time for the repositioning of these proteins. If there are any changes in these proteins during TJ opening, these alterations are not detectable by the conventional immunofluorescence.

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