Perturbation of potentially regulatable endoplasmic reticulum (ER) calcium stores with the Ca-ATPase inhibitor, thapsigargin (TG), perturbs the formation of desmosomes and tight junctions during polarized epithelial cell biogenesis, despite the development of cell contact. In a Madin-Darby canine kidney cell model for intercellular junction assembly, TG treatment inhibited the development of transepithelial electrical resistance (TER), a measure of tight junction assembly, in a dose-dependent manner. The TG-induced inhibition of tight junction assembly was paralleled by a defect in the sorting of the tight junction protein, ZO-1. An even more dramatic delay in sorting of the desmosomal protein, desmoplakin, was observed in the presence of TG. In addition, while both ZO-1 and desmoplakin-I in control cells were shown to become associated with the Triton X-100 insoluble cytoskeleton during intercellular junction assembly, prior treatment with 100 nM TG diminished this biochemical stabilization into the detergent-insoluble fraction, particularly in the case of ZO-1. Although spectrofluorimetric measurements in fura-2 loaded Madin-Darby canine kidney cells confirmed the occurrence of TG-mediated release of calcium from internal stores, total cytosolic calcium during junction assembly remained similar to untreated cells. Therefore, the presence of cytosolic calcium alone is not sufficient for normal intercellular junction biogenesis if intracellular stores are perturbed by TG. The results indicate the presence of calcium-sensitive intracellular mechanisms involved in the sorting and cytoskeletal stabilization of both tight junction and desmosomes and suggest a role for calcium-dependent signaling pathways at an early (possibly common) step in polarized epithelial biogenesis.

The ability of epithelial tissues such as the kidney and intestine to carry out vectorial transport of solutes and water is critically dependent on the maintenance of a permeability barrier to passive diffusion and upon the polarized distribution of transport proteins in the plasma membrane (Handler, 1989; Rodriguez-Boulan and Nelson, 1989; Simons, 1991). Despite the central role of intercellular junctions in epithelial physiology, much remains to be known about signaling pathways involved in junction regulation and biogenesis. The assembly of intercellular junctions has been studied in the intact embryo as well as in cultured cell models. In both settings, it has been proposed that intercellular junction formation is initiated by cadherin-mediated cell-cell contact and the formation of the adherens junction. This is closely followed by the assembly of desmosomes and tight junctions. Although classical signaling events have been shown to be important in the regulation of the assembly process, there is a scarcity of information regarding the precise role of these molecules in mediating specific assembly steps.

These issues are exceedingly difficult to address biochemically in the intact embryo where many different cell types are present. Moreover, in the intact embryo, the process of intercellular junction formation (even within a given tissue) does not occur in a precisely synchronous manner. MDCK cells in culture provide an excellent model for the assembly of intercellular junctions and the development of apical-basolateral polarity. MDCK cells maintained in low calcium media (LC media) lack cell-cell contact, intercellular junctions, and apical-basolateral polarization of lipids and protein (Cereijido et al., 1978; Gumbiner et al., 1988; Nigam et al., 1991; Siliciano and Goodenough, 1988; Stuart et al., 1994, Stuart and Nigam, 1995). Upon “switching” to normal calcium media (NC media), the cells rapidly develop characteristics of a polarized, tight, transporting epithelium. In many respects, the MDCK cell calcium switch recapitulates in vitro key events in epithelial morphogenesis (Rodriguez-Boulan and Nelson, 1989). The presence of a single cell type undergoing intercellular junction formation in a synchronous manner allows the process to be followed by immunocytochemical, biochemical, as well as physiological means.

We have previously observed global and local changes in intracellular calcium concentration ([Ca]) during the biogenesis of intercellular junctions (Nigam et al., 1992). In addition, we have demonstrated that chelation of intracellular calcium retards the assembly of tight junctions (Stuart et al., 1994). On the basis of these data and the work of others demonstrating that flux of calcium across the plasma membrane is not essential to tight junction formation (Gonzalez-Mariscal et al., 1990;...
Contreras et al., 1992), we have hypothesized that intracellular calcium is an essential part of the signaling cascade involved in intercellular junction biogenesis. This view is supported by data indicating that Ca\(^{2+}\)-dependent kinases, such as protein kinase C, play an essential role in tight junction formation (Nigam et al., 1991; Balda et al., 1993; Stuart and Nigam, 1995).

In epithelial and other cells, both the distribution of intracellular calcium stores and the nature of calcium movements between stores and the extracellular environment appears to be quite complex and is incompletely understood (Lyttton and Nigam, 1992). Interaction of a ligand with a specific membrane receptor often results in a biphasic increase in intracellular calcium. A fast initial release of Ca\(^{2+}\) from internal stores in response to inositol 1,4,5-triphosphate (IP\(_3\)) may be followed by a sustained phase of entry of extracellular calcium (Putney, 1987). The calcium-ATPase inhibitor, thapsigargin (TG), transiently activates this pathway, and as a result of the depletion of internal stores of calcium in the endoplasmic reticulum (ER), renders it unresponsive to further stimulation (Thastrup et al., 1990; Ghosh et al., 1991). Although in epithelial cells, there may be multiple inositol 1,4,5-triphosphate receptors both within the ER and elsewhere in the cell (Sharp et al., 1992; Bush et al., 1994), the TG-sensitive and experimentally inositol 1,4,5-triphosphate-releasable pools of intracellular calcium are believed to exist in the ER and largely overlap. Prior treatment with either agent quantitatively inhibits subsequent Ca\(^{2+}\) release from internal stores in response to the other agent (Thastrup et al., 1989). Thus, by virtue of its ability to selectively deplete a key regulated pool of intracellular calcium, TG is a uniquely valuable tool with which to assess the role of internal stores of calcium in tight junction biogenesis.

We now show that selective depletion of ER calcium stores prior to the initiation of cell-cell contact disrupts the biogenesis of desmosomes and tight junctions. Pretreatment with TG resulted in a marked inhibition of the development of transepithelial electrical resistance (TER), a late event in intercellular junction biogenesis. In addition, TG-sensitive calcium stores were shown to be necessary for intermediate sorting and biochemical stabilization steps in tight junction and desmosome biogenesis. The immunocytochemical and biochemical changes were observed to occur in the absence of any apparent effect on cell-cell contact or any significant change in whole cell calcium concentration. The data is consistent with an instructive role for intracellular calcium in the sorting and macromolecular assembly of junctional components.

**EXPERIMENTAL PROCEDURES**

**Materials—**Culture media was from Life Technologies, Inc. (Grand Island, NY). Plasticware was from Falcon (Lincoln Park, IL) except that Transwells were from Costar (Cambridge, MA). TG was obtained from Life Technologies, Inc. Anti-ZO-1 hybridoma R40.76 was the gift of Dr. Dan Goodenough (Harvard); the anti-E-Cadherin hybridoma, r1, was the gift of Dr. Barry Gumbiner (Memorial Sloan-Kettering); the anti-desmoplakin polyclonal antibody, NW6, was the gift of Dr. Kathleen Nelson (Northwestern).

**Cell Culture and TER—**Determinations of TER and maintenance of MDCK cells (ATCC) were performed as described previously (Stuart et al., 1994). Briefly, confluent monolayers of MDCK cells were incubated overnight in LC media, then 30 min in LC media containing concentrations of TG indicated in the figure legends, washed three times in LC media, and switched to NC media along with controls. The dose-response relationship (Fig. 1) indicated that a near maximal effect on TER was achieved with 100 nM TG. Therefore, further experiments employed 100 nM TG.

**Measurement of Intracellular Calcium—**Monolayers of MDCK cells grown on glass coverslips were incubated 45 min in LC containing 5 mM fura-2-AM (prepared without phenol red, vitamins, or amino acids) and spectrofluorimetric measurements of intracellular calcium performed as described previously (Stuart et al., 1994).

**Immunocytochemistry—**Monolayers of MDCK cells grown on glass coverslips were washed in phosphate-buffered saline and incubated in LC media as in TER determinations. The cells were pretreated with 100 nM TG, washed in LC media, and switched to NC medium. At the times indicated in the figure legends, the cells were processed for immunocytochemistry by standard techniques as described previously (Stuart et al., 1994).

**Transmission Electron Microscopy—**MDCK cells plated onto 12-mm Transwells at confluence were treated with 100 nM TG as described above. At the end of the treatment, the cells attached to the Transwell filter were processed for electron microscopy as described previously (Bush et al., 1990). Thick (1 μm) sections stained with toluidine blue were used for light microscopy. Thin sections were cut with a diamond knife, mounted onto Formvar-coated slotted grids (1 × 2 mm single hole), contrasted with uranyl acetate and lead citrate, and examined with an electron microscope.

**RESULTS**

Since TG is a highly specific and potent inhibitor of the ER Ca-ATPase and is known to selectively deplete calcium from this organelle, it is an extremely useful tool with which to investigate the role of ER calcium stores in cell regulation (Thastrup et al., 1989). We therefore pretreated MDCK cells with TG prior to the induction of cell-cell contact and monitored intercellular junction assembly by physiologic, immunocytochemical, morphologic, and biochemical means.

Depletion of internal stores of calcium by prior treatment with TG resulted in a dose-dependent inhibition in the development of TER, a quantitative measure of tight junction integrity (Fig. 1). Control cells reached maximal TER between 8 and 12 h, while MDCK monolayers treated with TG for 30 min prior to the induction of cell-cell contact displayed a dose-dependent reduction in the development of TER. The effect was significant even at very low (10 nM) concentrations and nearly maximal at 100 nM. Four hours after cell contact, cells pretreated with 100 nM TG achieved 15–20% of control TER, and an impressive effect was observed for longer than 12 h. Because 200 nM TG was not significantly more effective in inhibiting TER development, we employed treatment with 100 nM TG in subsequent studies.
Since internal stores of calcium derived from the ER (the TG-sensitive pool) appear to be important for calcium-mediated signaling within the cell and since depletion of these stores in other cell types is associated with a transient increase in intracellular calcium, we measured [Ca\(^{2+}\)] intracellularly using ratiometric fura-2-loaded MDCK cell monolayers during treatment with TG. In intact MDCK cell monolayers, treatment with TG resulted in a biphasic calcium transient. The biphasic calcium response to TG is consistent with that seen in other cell types following inhibition of ER calcium uptake by the Ca\(^{2+}\)-ATPase (Ghosh et al., 1991; Thastrup et al., 1989). The transient phase elevation in cytosolic calcium was no longer significant by 30 min after treatment with TG; the cytosolic [Ca\(^{2+}\)] was 23 nM in treated cells compared to 17 nM in control cells at this time point. Therefore, subsequent studies employed a 30-min period of TG treatment prior to initiation of cell-cell contact in order to control for whole cell intracellular calcium levels in treated and control cells.

Because we had previously observed significant global and local changes in intracellular calcium associated temporally with the formation of junctions during the MDCK cell "calcium switch" (Nigam et al., 1992), we investigated the effect of TG on intracellular calcium concentration during junctional biogenesis in this model. Prior treatment with 100 nM TG did not significantly alter the typical dynamics of intracellular calcium concentration observed after the initiation of cell-cell contact. In untreated cells, intracellular calcium concentration rose from ~20 to ~170 nM after a transient peak of approximately 400 nM (Fig. 2). Cells previously treated with 100 nM TG for 30 min also had a starting intracellular calcium concentration of ~20 nM which peaked and fell to a stable value of ~190 nM (Fig. 2), similar to untreated cells. Taken together with the TER results, this data suggested that the cell-signaling processes which are dependent upon intact TG-sensitive stores of intracellular Ca\(^{2+}\) are necessary for normal tight junction assembly and that maintenance of normal whole cytosolic Ca\(^{2+}\) levels is not sufficient for normal tight junction biogenesis if TG-sensitive stores are perturbed.

Since, in cell culture models, the biogenesis of intercellular junctions, including tight junctions, adherens junctions, and desmosomes, occur together at sites of cell-cell contact, it seemed possible that calcium (either directly or via the action of calcium-dependent protein kinases) might regulate the assembly of these intercellular junctions by affecting some common mechanism in their assembly. We therefore investigated the effect of ER calcium depletion on the sorting/redistribution of markers for tight junctions, adherens junctions, desmosomes, as well as the actin cytoskeleton. It has previously been shown that, prior to the establishment of cell-cell contact, two tight junctional proteins, ZO-1 and cingulin, are found primarily intracellularly (Gumbiner et al., 1988; Nigam et al., 1991; Sili-dano and Goodenough, 1988; Stuart et al., 1994; Stuart and Nigam, 1995). After cell-cell contact establishes, these proteins gradually appear on the lateral surface of the plasma membrane and, within approximately 2 h, begin to give a characteristic tight junction staining pattern by immunofluorescence. In like manner, markers for desmosomes and the adherens junction undergo a well defined redistribution after cell-cell contact establishes (Gumbiner et al., 1988).

Treatment with TG retarded the movement of ZO-1 (Fig. 3, compare bottom with top panel) from its intracellular location to the lateral plasma membrane during tight junction assembly. The difference was most notable 1 h after the initiation of cell-cell contact, at which point ZO-1 had largely sorted to the lateral surface of the plasma membrane in control cells, while those treated with TG displayed predominately intracellular staining. In addition, we observed that depletion of internal calcium stores by pretreatment with TG also markedly delayed the sorting of the desmosomal protein, desmoplakin (Fig. 3). As with ZO-1, the effect was most noticeable 1 h after the initiation of cell-cell contact at which time control cells consistently demonstrated more complete sorting of desmoplakin to the lateral plasma membrane. By 4 h, the staining pattern of desmoplakin in control and treated cells was indistinguishable (data not shown). In contrast to the defect in sorting of the tight junction proteins, the effect of TG on the redistribution of desmoplakin is specific to its desmosomal localization, as it still correctly localizes to desmosomes in the treated cells (Fig. 3).
junction protein, ZO-1, and the desmosomal protein, desmoplakin, we observed no effect on the accumulation at the adherens junction of the adhesion molecule, E-cadherin. In addition, minimal if any effect was observed on the reorganization of F-actin into the cortical actin ring in the setting of TG treatment (Fig. 3). Furthermore, the inhibition of intercellular junction formation did not appear to be the result of a failure of cell-cell contact (Fig. 4), thought to depend on an adhesive function intrinsic to E-cadherin. Thus, in addition to the apparently normal E-cadherin redistribution observed by immunofluorescence, we observed extensive and complete cell-cell contacts both by light and electron microscopy of transversely sectioned filters (Fig. 4). Nevertheless, it remains possible that some more subtle aspect of cadherin function was perturbed by treatment with TG which subsequently affected “downstream” events involved in tight junction and desmosome assembly (see below).

To gain insight into intermediate assembly steps that must occur between the arrival of a junctional protein and its incorporation into a final macromolecular assembly, we sought to analyze the process biochemically. One such assay of intermediate assembly is the Triton X-100 solubility of certain cytoskeleton-associated proteins, including many junctional proteins such as ZO-1, desmoplakin, and E-cadherin (Pasdar and Nelson, 1988; Stuart et al., 1994). The titration of these junctional proteins into a Triton-insoluble pool is generally believed to represent their association, directly or indirectly, with insoluble components of the cytoskeleton including actin microfilaments and/or intermediate filaments.

We therefore extracted monolayers of MDCK cells with a Triton X-100 containing buffer and determined the effect of TG on the titration of ZO-1, desmoplakin-I, and E-cadherin into the Triton X-100 insoluble pool during the establishment of intercellular junctions (Fig. 5). The biochemical stabilization into a Triton-insoluble fraction (R, for “residue”) during the formation of intercellular junctions was most impressive for ZO-1, but a similar effect was seen for desmoplakin-I (Fig. 5, left and center panels). Thus, the relative amount of ZO-1 in the soluble fraction (lane labeled E, for “extract”) decreased considerably in cells 6 h after contact (NC) as compared to uncontacted cells (LC). Pretreatment with 100 nM TG for 30 min prior to the initiation of cell-cell contact almost completely prevented the stabilization of ZO-1 even 6 h later (Fig. 4) despite nearly complete sorting of the protein by this time. In some experiments, TG treatment appeared to also prevent the biochemical stabilization of desmoplakin-I, but the effect was not as consistent or pronounced as with ZO-1 (Fig. 5B). The titration of E-cadherin into the insoluble fraction after cell-cell contact was less consistent and thus it was difficult to determine if TG prevented the biochemical stabilization of E-cadherin. Thus, the data indicate that calcium derived from the ER is necessary.
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for the association of ZO-1 with Triton-insoluble proteins; the data are also suggestive for a role of ER calcium in the association of desmoplakin-I with Triton-insoluble proteins.

Since ZO-1 exists in a complex (Balda et al., 1993; Stuart and Nigam, 1995) that appears to become associated with Triton-insoluble structures during the establishment of intercellular junctions, we speculated that solubilization of the intact complex and analysis of the size of this complex by rate zonal centrifugation might provide insight into the effect of TG treatment on macromolecular assembly of the junctional complex.

We therefore extracted MDCK cell monolayers with a modified RIPA buffer that has been useful for studying protein-protein interactions in the tight junction. The cell extracts were analyzed by 5–20% sucrose gradient centrifugation, SDS-PAGE, and immunoblotting. ZO-1 sedimented with an apparent molecular mass that ranged from its native 225 to ~800 kDa which is roughly consistent with the expected molecular mass of a previously identified multiprotein complex containing ZO-1, ZO-2, p130, and one or two yet to be identified proteins (Balda et al., 1993; Stuart and Nigam, 1995) (Fig. 6, top panel). Likewise, both E-cadherin and desmoplakin migrated in patterns consistent with monomers as well as the existence of larger complexes (Fig. 6, middle and bottom panels). Treatment with TG had no clear effect on the migration patterns of the three junctional proteins through the sucrose gradient. The sizing data taken together with Triton solubility data suggest that ZO-1 and DP-I associate with additional (insoluble, presumably cytoskeletal) proteins during the assembly of their respective junctions through a mechanism involving TG-sensitive intracellular Ca$^{2+}$ stores; however, at least under conditions commonly employed to solubilize intact ZO-1, DP-I, and E-cadherin containing complexes, we were unable to demonstrate an effect of TG on the association of these proteins into soluble macromolecular complexes.

**DISCUSSION**

On the basis of our previous finding that, during the MDCK cell calcium switch, impressive global and local increases in intracellular Ca$^{2+}$ occur during junction formation (Nigam et al., 1992), we have hypothesized that intracellular calcium is involved in signaling pathways leading to intercellular junction biogenesis. In addition, we had previously observed that buffering these changes in intracellular calcium with dimethyl BAPTA-AM disrupted several measures of tight junction assembly including the development of TER and the sorting of the tight junction protein ZO-1 (Stuart et al., 1994). However, these changes were observed in the setting of a reduced intracellular calcium concentration (the result of treatment with cell permeant calcium chelators), and it remained possible that a normal level of intracellular calcium was merely permissive for tight junction assembly. In the present study, the ER calcium-ATPase inhibitor, TG, serves as a unique tool with which to profoundly disrupt calcium-mediated cell signaling without significantly affecting whole cell calcium levels (Fig. 2).

We have now shown that treatment with TG prior to the initiation of cell-cell contact disrupts the biogenesis of tight junctions and desmosomes as evidenced by the inhibition of the development of TER (Fig. 1), as well as defects in the sorting (Fig. 3), and biochemical stabilization of junctional proteins into the cytoskeletal fraction (Fig. 5). Since both sorting and biochemical stabilization of junction proteins are steps thought to be distal to, and dependent on, the formation of productive cell-cell contacts, it remained possible that the effect of TG on both desmosome and tight junction formation was the result of a primary defect in cell adhesion. However, the immunocytochemical and biochemical changes induced by TG were observed to occur in the absence of any observable effect on the development of extensive cell-cell contacts by light and electron microscopy (Fig. 4) or on the accumulation of E-cadherin at the adherens junction (Fig. 3). Thus, the primary adhesive function of E-cadherin did not appear to be critically dependent on intact ER calcium regulation, although a subtle functional impairment cannot be excluded.

While the assembly of junctional proteins into large soluble macromolecular complexes was not detectably perturbed by TG under the solubilization conditions we employed (Fig. 6), the incorporation of ZO-1 and, to a lesser degree, desmoplakin, into Triton-insoluble structures required the presence of intact ER stores of calcium (Fig. 5). We have demonstrated that between 4 and 8 h after the calcium switch ZO-1 becomes relatively more resistant to extraction in Triton containing buffers (Stuart et al., 1994; Stuart and Nigam, 1995). In addition, we have shown that chelation of intracellular calcium during the calcium switch (but not in confluent monolayers) largely prevents the association of this tight junction component with the “cytoskeletal fraction.” However, the previous work left open the possibility that it was the mere presence of normal levels of cytosolic calcium that permitted a calcium-dependent association of ZO-1 with insoluble structures. The inclusion of calcium chelators such as EDTA in an extraction buffer can themselves increase the apparent solubility of ZO-1 (Anderson et al., 1988) and it remained possible that the chelators in the previous study produced an analogous physicochemical effect.

However, we have now demonstrated that a more subtle disruption of cell calcium with TG also largely prevented the association of ZO-1 with the Triton-insoluble cytoskeleton. The prevention of the biochemical stabilization of ZO-1 occurred in the absence of any significant difference in whole cell calcium concentration indicating that ER calcium stores play an important role in the association of ZO-1 with the cytoskeletal fraction. Despite large changes in the solubility of ZO-1 and DP with TG treatment, no obvious disruption of the actin-based cytoskeleton was observed in rhodamine-phalloidin stained cells (Fig. 3). However, it remains possible that a more subtle disturbance in the formation of the actin cytoskeleton underlies...
TG-induced solubility changes in ZO-1. TG-induced changes in the actin cytoskeleton would not be expected to effect the solubility of desmosomal proteins which are not thought to be primarily anchored to the actin-based network.

Although the titration of ZO-1 and desmoplakin into a Triton X-100 insoluble pool was dependent on an intact intracellular calcium regulatory mechanism, this decrease in solubility seems unlikely to be mediated by protein kinase-C. We have previously observed that treatment of MDCK cells with specific inhibitors of protein kinase-C during the formation of the junctional complex had unimpressive effects on the Triton solubility of these proteins despite clear-cut effects on the sorting of ZO-1 and development of TER (Stuart and Nigam, 1995). The present solubility data taken together with the immunocytochemical data imply diverging signaling steps in tight junction assembly. Thus, the sorting of tight junction proteins appears to be dependent on the action of both protein kinase C and intact internal stores of calcium; however, protein kinase C appears relatively uninvolved in the biochemical stabilization of at least one tight junctional protein (ZO-1), although its stabilization is clearly dependent on intact ER Ca\(^{2+}\) stores. Similarly, the sorting of desmosomal proteins appears to be impressively dependent on intact ER calcium stores; however, protein kinase C inhibition seemed to have little effect on desmosomal biogenesis, at least by immunofluorescence (Stuart and Nigam, 1995).

Since TG is known to selectively cause the release of calcium from ER stores, our results indicate that calcium derived from this compartment is involved in the sorting and biochemical stabilization steps in intercellular junction assembly. Based on current knowledge of cellular signaling events involving Ca\(^{2+}\), it would be expected that these intracellular stores would be most important in initiating any Ca\(^{2+}\)-dependent signaling events resulting from the establishment of cell-cell contact and perhaps critical for initiating calcium-dependent signaling pathways in epithelial morphogenesis in vivo. Conceivably, these stores are also responsible for the apparent localized change in intracellular Ca\(^{2+}\) which we had previously observed at sites of cell-cell contact (Nigam et al., 1992), which are also the sites of junctional biogenesis. Nevertheless, given the complexity of intracellular calcium stores in epithelial and other cells (Lyton and Nigam, 1992), particularly the inositol 1,4,5-triphosphate-sensitive pool(s) (Bush et al., 1994), and the even more complex secondary movements of Ca\(^{2+}\) (Putney, 1987), it is difficult based on this study alone to define the dynamics of Ca\(^{2+}\) involved in junctional biogenesis. However, the results of this study should help to further understand how signaling events activated upon cell-cell contact lead to the assembly of intercellular junctions.

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