Protein Kinase C Activation Increases Transepithelial Transport of Biologically Active Insulin

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Abstract

Protein kinase C activation leads to tight junctional leakiness and, consequently, to increased transepithelial (paracellular) solute flux across epithelial barriers. This leakiness is shown here to result in as much as a 20-fold increase in the transepithelial flux of insulin. Using an epithelial/fibroblast coculture model, this transepithelially transported insulin is shown to be biologically active. The 3T3 fibroblasts situated on one side of the epithelial barrier exhibited increased insulin binding and resulting DNA synthesis when the epithelial junctions were made leaky to insulin on the opposite side of the epithelial barrier. The dramatically enhanced permeability of macromolecules across epithelial cell layers undergoing protein kinase C activation may play a significant role in epithelial cancer, immunology, and drug delivery.

Introduction

PKC3 has a unique and central niche in signal transduction, due in no small part to its interdependence with calcium homeostasis and phospholipid turnover (1). Among its numerous actions, activation of PKC has been shown to result in leakiness of the tight junctional barriers surrounding epithelial and endothelial cells (2-11). A similar situation can arise from the overexpression of PKC isoforms within epithelia and endothelia (3, 12). This leakiness is generally reported as a decrease in TER, signifying enhanced permeability to sodium and chloride ions, or as an increase in the flux of d-mannitol (M). However, the activation of PKC in LLC-PK1 epithelia has been shown to increase the transepithelial flux of solutes as large as dextrans of M, 2,000,000 (13). Transepithelial flux of the protein mitogen EGF (M, 6,100) likewise increased almost 35-fold after the exposure of LLC-PK1 cells to the phorbol ester TPA (14).

The phorbol ester class of PKC activators are potent tumor-promoting agents, which, when repeated repetitively after the exposure of cells to a primary carcinogen, can significantly increase the number and size of tumors appearing in an epithelial tissue (15-16). It seems plausible that there might be a causal relationship between the effect of phorbol esters on epithelial tight junction permeability, as described above, and their role in epithelial carcinogenesis in such tissues as the urinary and gastrointestinal tract. In this specific study, we have asked whether phorbol ester-mediated PKC activation can increase the transepithelial flux of insulin, and if so, whether the enhanced insulin flux can bring about increased DNA synthesis in a subepithelial cell population.
TPA-INDUCED TRANSEPITHELIAL LEAKINESS TO INSULIN

Table 1 Transepithelial transport rates of insulin and mannitol across control and TPA-treated LLC-PK1 epithelial cell sheets

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>TPA-treated</th>
<th>Relative increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>0.012 ± 0.005</td>
<td>0.095 ± 0.017</td>
<td>7.9-fold</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.538 ± 0.152</td>
<td>11.782 ± 2.440</td>
<td>21.9-fold</td>
</tr>
</tbody>
</table>

Table 2 Unidirectional transport rates of 125I-insulin across control and TPA-treated cell sheets

<table>
<thead>
<tr>
<th>Condition</th>
<th>Apical to basal-lateral</th>
<th>Basal-lateral to apical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.100 ± 0.025</td>
<td>0.105 ± 0.018</td>
</tr>
<tr>
<td>TPA-treated</td>
<td>9.0 ± 2.0</td>
<td>9.4 ± 0.8</td>
</tr>
</tbody>
</table>

Results

When confluent, differentiated LLC-PK1 cell sheets were grown on permeable filters as described in “Methods and Materials” and exposed to 10^{-7} M TPA, the transepithelial PD immediately and irreversibly depressed from 1.5 mV to only 0.1 mV in less than 150 min, suggesting a rapid increase in the permeability to salts and water. However, an increase in the permeability to Na^+ and Cl^- says nothing definitive about permeability changes to solutes of higher molecular weight. We therefore measured in parallel the transepithelial (apical to basal-lateral) flux of [14C]mannitol and [125I]insulin, and [3H]thymidine in the permeability to Na^+CF, and thereby the transepithelial flux rates. As shown in Table 1, the apical to basal-lateral flux rates of [14C]mannitol and [125I]insulin were increased 7.9-fold, or as much as 21.9-fold, if the apical insulin concentration was increased to 10 μg/ml. The determination of these insulin flux values was performed only after gel filtration purification of the transepithelially transported [125I]insulin. This was necessary, because unlike o-mannitol, which cannot be metabolized by LLC-PK1 cells, and which has negligible affinity for their membrane transporters (18), insulin can be intracellularly degraded by renal epithelia (19–21). The [125I]insulin material coming across the LLC-PK1 barrier may therefore be either insulin or its degradation products. In experiments using 10 μg/ml insulin, gel filtration chromatography of the transepithelially transported radioactivity showed that the fraction of [125I]insulin associated with insulin rose from 13% for control cell sheets to 42% for cell sheets treated with TPA. This difference presumably reflects the lack of degradative enzymes in the paracellular pathway.

Comparing the flux of insulin across TPA-treated cell sheets at two different insulin concentrations provides additional evidence for transepithelial insulin flux proceeding via a paracellular pathway. With a 100-fold increase in the apical insulin concentration (and therefore a 100-fold increase in the insulin concentration gradient across the cell sheet), the flux of insulin increases from 0.095 to 11.782 pmol/h/cm^2 (Table 1). This approximately 100-fold increase would be the expected relationship for a solute that crosses the cell sheet by simple diffusion. However, for the control cell sheets, a 100-fold increase in the insulin concentration gradient raises the insulin flux from 0.012 to 0.538 pmol/h/cm^2, an increase of less than 45-fold. This smaller-than-expected increase could be explained by a significant transcellular receptor-mediated route for insulin flux across the control cell sheets. However, the paracellular pathway would involve only simple diffusion, and therefore it would increase proportionately to the increase in concentration gradient.

A comparison of apical to basal-lateral versus basal-lateral to apical insulin fluxes provides further support for a paracellular route for transepithelial insulin flux across TPA-treated cell sheets. If the primary route of transport is paracellular, and therefore simple diffusion, one would expect to see bidirectional symmetry in the two fluxes. However, if a significant receptor-mediated transcellular route existed, one would predict that this would function vectorially in one direction and would therefore result in asymmetry in the transepithelial flux rates. As shown in Table 2, the apical to basal-lateral flux of insulin was not significantly different than the basal-lateral to apical flux for both control cell sheets and TPA-treated cell sheets.
To further determine if the insulin entering the basolateral fluid compartment was biologically active, its ability to stimulate DNA synthesis in the basolateral 3T3 cells was observed. For these experiments, the basolateral fluid compartment contained serum-free α-MEM supplemented with 200 ng/ml EGF and 25 μg/ml transferrin. In separate experiments, we determined that this EGF/insulin culture medium provided only a very low level of DNA synthesis by the fibroblasts. The apical fluid compartment contained serum-free α-MEM supplemented with 100 μg/ml insulin. Diffusion of insulin into the basal-lateral fluid compartment would then result in an EGF/insulin culture medium in the basolateral compartment that, in separate studies, provided for a greater than 10-fold higher rate of DNA synthesis by the fibroblasts than was seen with only the EGF/insulin culture medium. As shown in Fig. 3, [3H]thymidine incorporation into the DNA of 3T3 fibroblasts in serum-free medium was extremely low. In a serum-free medium supplemented with EGF and transferrin, there was a significant increase. The addition of EGF, transferrin, and insulin then resulted in another very dramatic increase in [3H]thymidine incorporation. If the 3T3 cells were in an EGF/insulin-supplemented medium into which the Falcon 3102 filter rings with attached LLC-PK1 cell sheets were placed, this did not lead to a significantly greater level of DNA

To next ascertain that the insulin that diffused across the TPA-treated epithelial cell sheets retained biological activity, a target cell population was placed in the basal-lateral fluid compartment into which the insulin was diffusing. Specifically, Balb-c-3T3 fibroblasts were cultured in dishes into which were later placed the permeable filter rings containing the LLC-PK1 cell sheets (Fig. 1). This gave rise to two fluid compartments, one above the epithelium, in which the insulin was added, and one below the epithelium, in which the fibroblasts were growing. For a given experiment, LLC-PK1 cell sheets were cultured on permeable filter ring assemblies in separate dishes in which they were treated with control culture medium or medium containing the phorbol ester PDBU at 10−7 M for 2.5 h at 37°C to increase tight junction permeability. Control and PDBU-treated cell sheets were then rinsed three times with control medium and transferred to 6-well culture dishes containing adherent Balb-c-3T3 fibroblasts. The LLC-PK1 cell sheets (with the apical 125I-insulin) were coincubated with the 3T3 cells for 90 min at 37°C, followed by removal of the filter ring inserts, rinsing away unbound 125I-insulin at 4°C, and physically scraping the 3T3 cells for determining bound radioactivity. As shown in Fig. 2, if the LLC-PK1 cell sheet is first made leaky by exposure to PDBU, the 125I-insulin binding to the 3T3 cells is increased by over 20-fold. Specificity of 125I-insulin binding was confirmed by adding 200 μg/ml unlabeled insulin to the basolateral fluid compartment. The amount of 125I-insulin bound to the 3T3 cells, for the condition of PDBU-treated LLC-PK1 cell sheets, was reduced almost to control levels.

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Discussion

Using an epithelial/fibroblast coculture model, this study yielded two major findings: (a) phorbol esters can increase transepithelial permeability to insulin by at least 20-fold; and (b) this increased flux results in the intact transit of a biologically active growth factor into the equivalent of a submucosal compartment in which it can stimulate DNA synthesis in a fibroblast cell population. Using a cell culture model to simplify the architecture of an epithelial tissue, we therefore show that the activation of PKC in epithelium can allow for significantly increased transepithelial flux of biologically active molecules into tissue compartments from which they are normally sequestered. This result is supported by earlier findings showing that phorbol esters increase the epithelial tight junction permeability of electron-dense dyes such as ruthenium red, as well as dextrans as large as M, 2,000,000 (17).

If the paracellularly transported growth factors moving from the apical to basal-lateral fluid compartment have access to subepithelial fibroblasts, they will also have access to the basal-lateral surfaces of the epithelial cells themselves. The intrinsic polarity of the epithelial cells results in their growth factor receptors being normally sequestered on the basal-lateral cell surface (22, 23). Normally, any growth factors that are compartmentalized in the luminal fluid will have access only to the apical cell surface. Increased paracellular permeability may therefore allow such growth factors to influence DNA synthesis by the epithelia themselves, as well as cell types situated below the epithelial barrier.

The high concentrations of EGF in the urine or of EGF, amphiregulin, and Cripto in the gastrointestinal lumen are two examples of the presence of growth factors at very high levels in epithelial luminal fluid compartments (24–28). These are levels far in excess of those seen in the bloodstream. In the urinary and gastrointestinal tracts, such levels arise from the synthesis of growth factors by the epithelia themselves, followed by their vectorial secretion into the lumen (24, 29). The presence of growth factors at high levels within luminal fluid is speculated to promote tissue regeneration at both the moment and the site of any physical wounding of the epithelium (30–33).

However, within the context of the two-stage model of epithelial carcinogenesis, we speculate that if an initiated focus of epithelial cells is placed under chronic promotional stimulus by PKC-activating agents such as phorbol esters, one of the results will be chronic tight junctional leakiness at this site. Such leakiness, moreover, need not arise only from a direct effect on tight junctional proteins. The proven association of the Adenomatous Polyposis Coli tumor suppressor protein with the cell adhesion/cytoskeletal protein β-catenin makes for a link between mutational-derived epithelial cancers and defective intercellular junctions (34). For tissues such as the urinary and gastrointestinal tracts, this should then result in chronic growth factor penetration onto the basal-lateral cell surface and into the interstitial fluid compartments below the epithelium.

Fig. 4. Potential outcomes of tight junctional leakiness to luminal growth factors. If the epithelial barrier becomes leaky to growth factor proteins in the luminal fluid compartment, effects can be produced on the nonepithelial cell populations in the interstitial compartment and/or the epithelium of the barrier itself. The latter situation follows from the fact that receptor sites on the basal-lateral surface of the epithelial cells are normally shielded from the effects of luminal growth factors by the intrinsic polarity of the epithelium and the tight junctional barrier between individual cells.

1. Luminal growth factor, X, accesses and activates basal-lateral receptors, directly leading to epithelial growth kinetic changes.

2. Luminal growth factor, X, accesses and activates receptors on cells in interstitium (fibroblasts, endothelia, etc.) altering their growth kinetics.

3. In response to X, interstitial target cells synthesize and secrete Y, which then acts upon epithelium.
As shown in Fig. 4, at least three outcomes are then possible: (a) a direct effect of these penetrating growth factors on the epithelia themselves by now having access to receptor sites on the basal-lateral cell surface; (b) a direct effect of the growth factors on various cell populations in compartments under the epithelium; and (c) a paracrine response whereby the newly stimulated cells in the interestitium now produce their own growth factors/cytokines that will then act on the epithelia. The biomedical implications could be much wider than epithelial carcinogenesis. The significance of such paracellular leakiness could be just as important in immunology and pharmacology, because conditions could then exist for unwanted antigen presentation across epithelial barriers (leading to chronic inflammatory conditions) or targeted protein and oligonucleotide drug delivery across epithelial barriers.

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References

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