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The phorbol ester, TPA, increases transepithelial epidermal growth factor flux

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Exposure of cultured kidney epithelial (LLC-PK_1) cell sheets to 10^{-7} M TPA, a potent tumor promoter and activator of protein kinase C, initiates within minutes a drop in the transepithelial voltage across these sheets. This fall in potential difference correlates with an over 40-fold increase in the transepithelial flux of 1 mM D-mannitol, suggesting that the intercellular junctions have become leaky. Dual labeling experiments with 1 mM D-[^{14}C]mannitol and 10 nM ^{125}I-EGF show that after promoter treatment, a 7-fold increase in net ^{125}I flux accompanies the increase in mannitol flux. Gel filtration and gel electrophoresis indicate that for control cell sheets only 15% of the transited ^{125}I is actually EGF, whereas with TPA-treated cell sheets, 60% of the ^{125}I which passed across is EGF. These percentages permitted determination of actual EGF flux values, and show that TPA treatment engenders a 35-fold increase in transepithelial EGF flux. Diacylglycerols also increase the junctional permeability of these cells, thereby suggesting the involvement of protein kinase C.

Tight junction; Tumor promoter; Phorbol ester; Diacylglycerol; (Epithelium; LLC-PK_1 cell)

1. INTRODUCTION

Most current work with phorbol esters centers on the cell and molecular level, specifically on understanding the interplay of tumor promoters in the naturally occurring signal transduction mechanisms involving protein kinase C, inositol phospholipids, diacylglycerols and inositol phosphates [1]. However, since normal growth regulation as well as carcinogenesis occurs in vivo within the context of tissues and organs, examining the effects of tumor promoters on the tissue level of organization may offer some new insights.

Epithelial cell cultures which retain polarity and form an intact epithelium upon reaching confluence offer a unique means of addressing such questions with a simplified 'tissue' model [2]. Culturing these polar cells on permeable filters permits examination of flux across the cell sheet between two fluid compartments, one of the more basic functions of any epithelium in vivo. Particularly useful in this regard is the LLC-PK_1 renal epithelial cell line, whose merits are detailed in a recent review [3].

In a related epithelial cell line, MDCK, it was observed several years ago that TPA decreased the electrical resistance across these cell sheets, indicating that the occluding junctions had been made leaky [4]. By means of simultaneously examining the electrical gradients across the cell sheets, and also the transepithelial flux of D-mannitol, the occluding junctions between LLC-PK_1 cells have been demonstrated to be made leaky by phorbol ester tumor-promoting agents [5]. D-Mannitol has been demonstrated to traverse these cell sheets by passing between cells, i.e. through tight junctions, and is therefore a useful probe for these types of studies [6]. In another polar epithelial cell line, T_{84}, the transepithelial flux of mannitol has been shown to be closely correlated

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to occluding junction structure, as seen in freeze-fracture electron micrographs [7].

Because there is evidence that in vivo carcinogenesis is associated with partial breakdown of epithelial occluding junctions [8], we wished to extend the observations which were made on low-M_r molecules such as D-mannitol, by testing the flux of higher-M_r molecules of known relevance to growth regulation. Epidermal growth factor (EGF) was chosen for its well-documented relevance to growth regulation [9], its relatively small size (M_r ~6100) among the known protein growth factors, and data indicating that it cannot readily cross epithelial barriers in vivo [10].

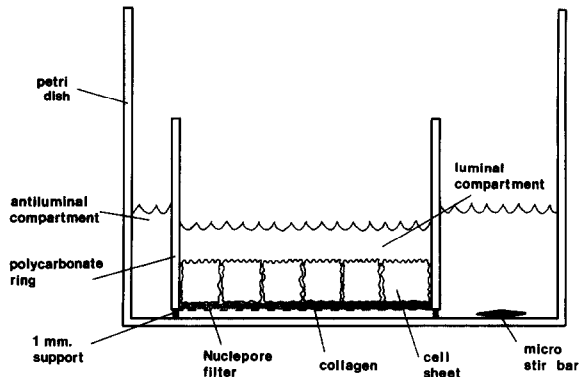


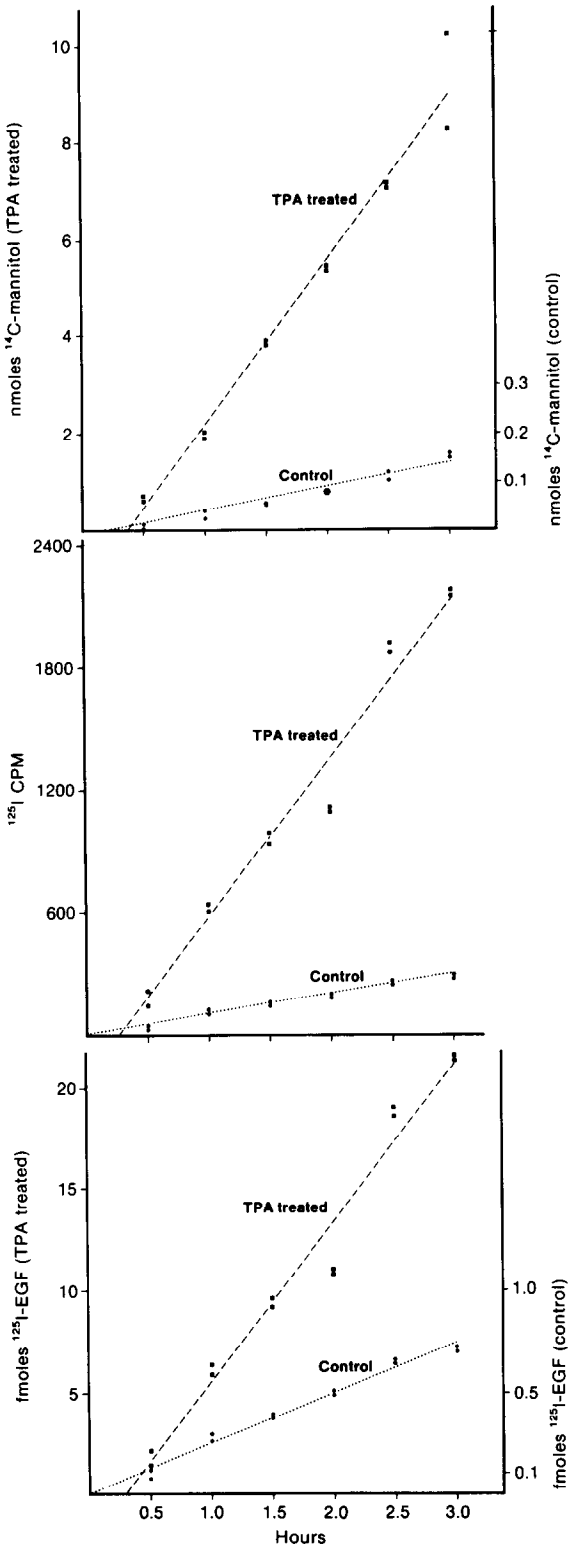
Fig.1. Incubation dish used in studying transepithelial fluxes across LLC-PK$_1$ cell sheets.

2. MATERIALS AND METHODS

For the determination of transepithelial fluxes, the cell culture and basic analytical procedures described by Mullin and O'Brien [5] for fluxes of D-mannitol were followed. Briefly, approx. 2 h before initiating fluxes, cell sheets (in filter-ring assemblies [5,11]) were refed (apical and basolateral compartments) with fresh medium [alpha MEM (Hazleton) with 10% fetal bovine serum (HyClone Labs)], with or without 10^{-7} M TPA (Chemicals for Cancer Research, Inc.), and incubated at 37°C in a 5% CO_2 atmosphere. The electrical potential difference (apical negative) was then monitored until the potential differences across those cell sheets treated with TPA fell to 0.3 mV or less, from control values averaging 1.0 mV. The electrical potential difference across control cell sheets remained stable throughout this period. Cell sheets in their ring assemblies were then rinsed three times in room-temperature morpholinopropanesulfonic acid-buffered saline [5] and then placed on 1-mm supports in 100-mm dishes for the flux studies. Saline without mannitol or EGF was placed in the apical compartment and saline containing both 1 mM D-[^{14}C]mannitol (ICN Radiochemicals) and 10 nM ^{125}I-EGF was placed in the basolateral compartment. The filter-ring assemblies in the 100-mm dishes containing micro-stir bars were then placed on stirring motors and the flux was followed at 25°C (see fig.1). Every 30 min, 25 μl samples were taken in duplicate from the apical compartment for analysis by liquid scintillation counting ([^{14}C]mannitol) and gamma counting (^{125}I). Duplicates were performed within each experiment and results were then confirmed by running a duplicate experiment on a different passage level of cells (passage levels ranged from 185 to 200). Unlabeled EGF (mouse submaxillary) was purchased from Collaborative Research. ^{125}I-labeled EGF (human recombinant) was a gift from Dr Ulrich Rodeck of the Wistar Institute.

It was decided to examine basolateral to apical flux of EGF instead of the reverse because of the geometry of the cell system. If a tracer molecule, evenly distributed through the basolateral compartment, traverses the occluding junction into the apical compartment, it enters a space where its free diffusion is relatively unimpeded. However, in the reverse scenario, if a tracer molecule, evenly distributed through the apical compartment, should cross the occluding junction, it enters the restrictive intercellular space, where its free diffusion into the total basolateral compartment may be impeded, and either backflux across the junction becomes significant, or binding to receptors in the intercellular space reduces the perceived flux.

For purposes of analyzing the ^{125}I which transited the cell sheets, 1-ml samples were withdrawn from the apical compartment at the end of the 3 h flux period. These samples were analyzed by gel filtration using a 30 cm × 9 mm column (Pharmacia) of Sephadex G-25. Blue dextran (M_r 2 × 10^6) was used as a marker of void volume. ^{125}I-EGF eluted in the void volume and cofractionated with blue dextran.[^{14}C]Tyrosine and phenol red were also used as markers.



As a further check to identifying that portion of ^{125}I which was EGF, gel-filtration fractions which cofractionated with Blue dextran on Sephadex G-25 were further analyzed by gel electrophoresis. The relatively low M_r of EGF and the necessarily lower M_r values of any degradation products necessitated the use of a 25% acrylamide-bisacrylamide gel. Under constant voltage (100 V), the gels were run for 18 h. Gels were stained with Coomassie brilliant blue R and then dried under vacuum. The dried gel was autoradiographed against Kodak X-Omat film at −70°C.

3. RESULTS

The appearance of D-mannitol in the apical compartment was linear over the 3 h period, in both control and TPA-treated conditions (fig.2, top panel). The sharp increase in the slopes of the lines indicates a more than 40-fold increase in the flux of D-mannitol across the cell sheets treated with TPA. As shown in table 1, the D-mannitol flux values rose from an average of 1.2 to 51.6 nmol/h per cm^2, as a result of TPA treatment. TPA also caused an increase in the rate of appearance of ^{125}I in the apical compartment. However, unlike D-mannitol which is non-metabolizable, EGF is subject to receptor binding, internalization and degradation, with consequent release of ^{125}I-tyrosine and other degradation

Fig.2. (Top) Appearance with time of D-[^{14}C]mannitol in the apical compartment after transiting the cell sheets. Methodology is described in the text. Each point represents a sample taken at the time indicated from the apical compartment of one of two separate cell sheets, for both control and TPA-treated. Note the use of an expanded ordinate for the TPA-treated set of samples. (Middle) Appearance with time of ^{125}I in the apical compartment after transiting the cell sheets. Conditions are as described above. Data represent net ^{125}I which would include both EGF as well as degradation products. (Bottom) Appearance with time of ^{125}I-EGF in the apical compartment after transiting the cell sheets. Again note the use of an expanded ordinate for the TPA-treated cell sheets. These are data derived from data of the middle panel using the information obtained in fig.3 showing the percent of transited ^{125}I which was actually EGF, and correcting for that percentage of ^{125}I in the incubation saline (top panel, fig.3) which was not EGF.

products into the apical compartment. These products, as well as intact EGF which transited through the junctions or across the cells, are both represented in the net ^{125}I appearing in the apical fluid. As shown by gel filtration using Sephadex G-25 (section 2), the ^{125}I in the incubation saline introduced at the beginning of the experiments was predominantly EGF (72%), with a small percentage of material cofractionating with tyrosine (13%), likely representing auto-degradation products of the EGF (fig.3, top panel). Gel-filtration analysis of the ^{125}I appearing in the apical compartment of control cell sheets shows the opposite profile (fig.3, middle panel). On average, over 50% of this material cofractionated with tyrosine, while only 15% cofractionated with EGF, indicating the relative inability of EGF to cross control cell sheets without undergoing degradation. Those cell sheets which had been treated with TPA manifest a quite different profile (fig.3, bottom panel). 60% of the ^{125}I which crossed these cell sheets cofractionated with EGF, suggesting that phorbol ester treatment allowed for significant quantities of intact EGF to cross cell sheets. The identity of the ^{125}I cofractionating with EGF was further checked by performing gel electrophoresis on those specific fractions, as described in section 2. A single band in the autoradiogram, comigrating with known EGF, was observed for the case of the void volume fractions of the ^{125}I-EGF applied to the cells, and the ^{125}I-EGF which crossed the control and TPA-treated cell sheets.

The above results allowed determination of specific fluxes of EGF from the rates of appearance of ^{125}I shown in fig.2 (middle panel). These actual EGF fluxes are given in table 1, and the rates of appearance of EGF are shown graphically in fig.2 (bottom panel). As can be seen the flux of EGF increased by almost 35-fold after cell sheets had been treated with tumor promoters.

Although TPA has been shown not to cause changes in the fluidity of artificial membranes [12], it remained possible that the TPA-induced junctional effects demonstrated here may be due to induced fluidity changes in the cell membrane, and

Table 1

Transepithelial flux values of 1 mM D-[^{14}C]mannitol and 10 nM ^{125}I-EGF across control and TPA-treated LLC-PK$_1$ cell sheets, at 25°C

	Control	TPA-treated
D-Mannitol	1.2 ± 0.3	51.6 ± 3.2
EGF	3.4 ± 0.6	123.1 ± 2.4

Values are given in nmol/h per cm^2 for D-mannitol and fmol/h per cm^2 for EGF, ± the range of two measurements of the apical compartments of two ring assemblies. The actual fluxes are plotted in fig.1. The values given here represent the average of the slopes of the lines obtained by linear regression

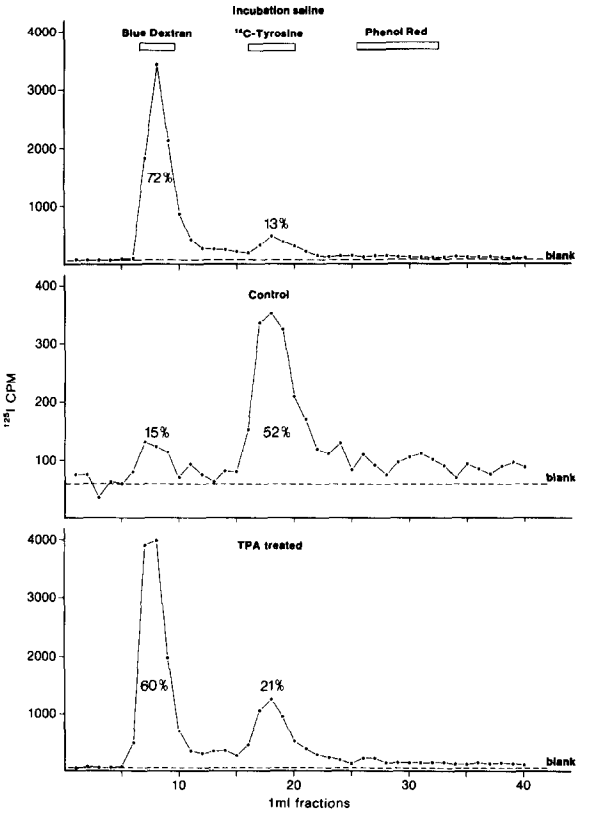


Fig.3. Analysis of the composition of the ^{125}I which transited the cell sheets. (Top) Fractionation pattern of the incubation saline applied to the basolateral compartment. 72% of the radioactivity was determined to be EGF. (Middle) Apical saline from control cell sheets, in which only 15% of the radioactivity existed as EGF, while 52% represented lower-M_r products cofractionating with tyrosine. (Bottom) Apical saline from TPA-treated cell sheets, where 60% of the radioactivity existed as EGF, but only 21% was apparent breakdown products cofractionating with tyrosine.

Table 2

Transepithelial flux of 1 mM D-[^{14}C]mannitol across control and diacylglycerol-treated cell sheets

	Control	Diacylglycerol-treated
D-Mannitol	1.7 ± 0.8	7.9 ± 0.3

The methods are identical to those reported in the text, except that cells were pretreated with 0.2 mM 1,2-dioctanoylglycerol instead of TPA. Results are given in nmol/h per cm^2 ± the range of two measurements. As in table 1 this experiment was repeated, again with duplicate cell sheets of a different passage level, to confirm results

not to specific effects of TPA on protein kinase C and subsequent protein phosphorylations. Moreover, recent evidence indicates that certain cellular effects of phorbol esters may not be mediated through the protein kinase C system [13]. Therefore, the effects on junctional permeability of a potent diacylglycerol, 1,2-dioctanoylglycerol, were studied. When cell sheets were exposed to 0.2 mM 1,2-dioctanoylglycerol, the electrical gradient across the cell sheet began to dissipate within 15 min at 37°C as in the case of TPA treatment. When fluxes of D-mannitol were studied as described previously, mannitol flux increased approx. 5-fold (table 2). Similar results have been obtained with another diacylglycerol, 1-oleoyl-2-acetylglycerol (unpublished). This similarity to phorbol ester treatment suggests that the changes in junctional permeability are being mediated through the protein kinase C signal transduction system.

4. DISCUSSION

Although the exact mechanism of EGF transit across the LLC-PK$_1$ cell sheets is unknown at present, pretreatment with TPA yields significant increases in the transcellular movement of this growth factor. Since mannitol, whose flux is similarly increased, is known to traverse these cell sheets by diffusion through the junctions [6], a similar phenomenon may account for the increased EGF flux.

The situation of a preneoplastic cell within a stratified epithelium may be viewed from a new perspective in light of these findings. Sequestered by the junctional bands of neighboring cells, such a cell may have its proliferative potential raised if protein growth factors are allowed freer access to the cell. This may represent a new nuance to the perceived action of tumor promoters, and indicate important effects at the tissue, as well as the cellular, level. Also, there may be an enhancement of tumor invasiveness once junctional strands of an epithelial or endothelial barrier are disrupted, judging from the reported correlation between transepithelial migration of neutrophils and the resistance of the epithelial layer [14].

Since this regulation of transepithelial permeability is apparently not caused specifically by tumor promoters, but more generally by activation of protein kinase C, the significance of these observations extends beyond carcinogenesis, into normal growth regulation, with wide application. If, for example, growth factors or other protein hormones are normally unable to cross the blood brain barrier [10], the effects of protein kinase C activation in the brain capillary endothelia or the choroid plexus epithelia might have implications in the field of neurobiology. Finally, the phenomenon of autocrine growth regulation in the kidney [15] may be particularly affected by transepithelial permeability to growth factors if autocrine growth factors are in certain instances not being released precisely at the cell surface where their receptors are located.

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