Evaluation of the Caco-2 Monolayer as a Model Epithelium for Iontophoretic Transport

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Purpose. To assess the Caco-2 monolayer as a model for iontophoresis of drugs across a model epithelium.

Methods. The apparent permeability co-efficient (Papp) of mannitol, thyrotrophin releasing hormone (TRH), dexamethasone and a range of sizes of fluorescein isothiocyanate (FITC) dextrans across Caco-2 monolayers was measured under passive and electrically stimulated conditions. Trans-epithelial electrical resistance (TEER) was determined throughout. Transmission electron micrographs (TEM) of the monolayers were taken. Confocal laser scanning microscopy (CLSM) was used to visualize the iontophoretic transport route of FITC-Dextran (MW = 20 kDa) across a Caco-2 monolayer.

Results. Application of 14.3 μ-Eq.cm^{-2} across the monolayer evoked a transient drop in TEER. The drop in TEER was accompanied by statistically significant increases in fluxes of all the agents in the mucosal to serosal direction except for FD-70. TEM of test samples exhibited tight junction dilatation, in addition to intracellular vacuolisation. The iontophoresis of FD-20 was visualised with confocal laser scanning microscopy and was localised in paracellular spaces of the monolayer.

Conclusions. The fluxes of mannitol, TRH, dexamethasone, FD-4, FD-10 and FD-20 across the Caco-2 monolayer were significantly enhanced when electric field was applied. The iontophoretic effect appeared to be directly upon tight junctions

KEY WORDS: iontophoresis; electro-osmosis; permeability; tight-junction; Caco-2.

INTRODUCTION

The human colon adenocarcinoma cell line, Caco-2 (1), forms a polarised monolayer with a well defined brush border on the apical surface and tight cellular junctions (2). Reproducibility and a longer capacity to maintain resistance values after differentiation confers significant advantages to the Caco-2 model over other *in vitro* models used for absorption studies. The Caco-2 model contains the three major permeability barriers to the absorption of drugs; i) the unstirred water layer, ii) the junctional complex between the cells and iii) polarized cell membranes. Consequently, Caco-2 cells have been used extensively to model intestinal absorption (3). Caco-2 cells do not however support a mucus layer (4). Results to date indicate that the Caco-2 model can be used to study the relative contribution of the paracellular and transcellular routes to the transport of drugs. Passive flux characteristics of drugs across the Caco-2 monolayer have shown a correlation with human oral bioavailability and so the Caco-2 model is a valuable tool for screening rank order of permeation of oral drug delivery systems (5).

Systemic availability of hydrophilic and/or macromolecular drugs, particularly peptides, administered perorally is often too low to have any therapeutic effect (6). Factors that can decrease bioavailability include low solubility, chemical instability in the lumen of the gut, high gastrointestinal metabolism and poor membrane permeability. (7). Chemical enhancers have been used in an attempt to modulate the absorption barrier. Examples include medium chain fatty acids (MCFA). For example C8, C10, and C12 when administered at a concentration in the vicinity of the critical micelle concentration for each MCFA, gave comparable increases in epithelial permeability enhancement of mannitol across Caco-2 monolayers (approx. 8-fold) (8). The chelating agent disodium ethylenediaminetetraacetate (EDTA) has been shown to increase the permeability of PEG4000 to 14 times the control compared to the MCFA C10 3.5 fold increase (9). It is evident from this that manipulation of the epithelial barrier, particularly the tight junction complex of the paracellular route, can be a means of improving the systemic availability of poorly absorbed molecules such as hydrophilic compounds or small peptides. It is not possible to extrapolate from these experiments anything about the safety and tolerance of treatments or procedures when applied *in vivo.*

Iontophoresis is defined as the process of increasing the penetration of solute molecules into or through a tissue by the passage of a direct electric current through an electrolyte solution containing the ionic molecules to be delivered, using an appropriate electrode polarity (10,11). Previously iontophoresis was shown to modulate drug absorption in rat colonic excised tissue (12). The flux of mannitol and TRH across intact colonic epithelium in vitro was enhanced when current was applied with a direct linear correlation between current application and the apparent permeability coefficient (Papp). The aims of the present study were to use Papp measurements to evaluate the Caco-2 cultured cell line as a model epithelium for iontophoretic transport of drugs and to compare the iontophoresis of a hydrophobic and a hydrophilic molecule across the epithelial layer. The permeability of a series of FITC-Dextran molecules ranging in molecular weight from 4–70kD was measured across Caco-2 monolayers to determine the size exclusion limit for the system. Transmission electron microscopy was used to analyse effects on the cell monolayer after the applied current and to visualise any distinct changes after a forty-minute recovery period. Confocal laser scanning microscopy was employed to ascertain the route of transport of the iontophoretic current.

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ABBREVIATIONS: Ci, Curie; CLSM, confocal laser scanning microscopy; DHPE, dihexadecanoylglycerophosphoethanolamine; F, Faraday constant; FD, fluorescein dextran; FITC, fluorescein isothiocyanate; GIT, gastrointestinal tract; KDa, kilodalton; MCFA, medium chain fatty acids; Papp, permeability coefficient; PD, potential difference; Q, charge; SCC, short circuit current; TEER, transepithelial electrical resistance; TEM, transmission electron microscopy; TRH, thyrotrophin releasing hormone; Ωhms.

MATERIALS AND METHODS:

Materials

^{14}C-Mannitol (51.5 mCi/mmol), ^{3}H-TRH (74 Ci/mmol) and ^{3}H-Dexamethasone (49.5 Ci/mmol) were purchased from NEN Life Science Products Ltd (Boston, USA). Cell culture media were obtained from Gibco (Paisley, Scotland). FD-4-, 10-, 20-, and 70-kDalton, DAFCO, EGTA, D-glucose, K PIPES, $NaB_4O_7$and paraformaldehyde were from Sigma (St. Louis, USA). Texas Red DHPE was from Molecular Probes (Leiden, Netherlands). All other chemicals were from Riedel de Haen (Seelze, Germany). Dual Voltage Clamp 1000 were from WPI (East Sussex, England) and the Sweetana-Grass side by side diffusion chambers were from Precision Instruments, Tahoe City(USA).

Methods

Permeability Experiments in Caco-2 Cells

Caco-2 cell monolayers, cultured in Dulbecco's Modified Eagles Medium (DMEM) 4.5 gL^{-1} glucose supplemented with 1% (v/v) non-essential amino acids, 10% Foetal Calf Serum, 1% penicillin/streptomycin at 37°C and 5% CO_2 in 95% relative humidity. Cells were grown and expanded in normal tissue culture flasks and passaged once they attained 100% confluence. Caco-2 cells were seeded on Transwell filters (Costar, 6.5mm diameter, 0.4μm pore size) at a density of 5×10^5 cells/cm^2 and incubated in 48 well culture plates with a medium change every second day. Confluent monolayers between day 20 and day 30 post seeding on filters were used for the transepithelial transport studies. The confluent Caco-2 monolayer was mounted between two identical halves of a Sweetana-Grass diffusion chamber (Precision Instruments, Tahoe City, USA). The monolayer was bathed (10ml on either side) with a Krebs-Hensleit physiological solution (113mM NaCl, 4.7mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 12.1 mM glucose, 25mM $NaHCO_3$, 1.9 mM $CaCl_2.2H_2O$) and maintained at 37°C. The solution was continuously gassed with 95% O_2-5% CO_2 to maintain the pH at 7.4, to oxygenate the cell monolayer and to provide a homogeneous circulation of the bathing fluids by the gas lift system.

Iontophoretic Protocol

The experimental design used to investigate for absorptive flux enhancement due to imposed current conditions comprised three consecutive periods of 20 minute duration (c.f. Fig. 1). The monolayer was studied under open circuit during basal and recovery periods. During the test period the monolayer was current clamped and a current applied that would transfer a charge of 14.3 μ-Eq.cm^{-2} (1.2 mA/cm^2) across the cell monolayer. Charge (Q) being transferred across the epithelium was calculated for each experiment using a Maclab data acquisition system and its value in equivalents is calculated by Q/F where Q = charge in coulombs [Q = current (amperes) * time (sec)] and F = Faraday's constant (96,500 Coulombs/mol). Ag/AgCl electrodes were used to pass and monitor current using a standard voltage clamp apparatus (World Precision Instruments) and the paracellular flux marker ^{14}C-mannitol was used to indicate any absorptive flux enhancement. At time zero, 3.0 μCi [^{14}C]-Mannitol was added to the apical side of the diffusion chamber. 100μl apical samples and 1000μl basolateral samples were taken at 20 minute intervals, which were replaced by warmed physiological solution. ^{14}C radioactivity in the samples was determined using a liquid scintillation counter (WALLAC, System 1409). The permeability co-efficient (Papp) was measured for mannitol for each sampling period using the following equation: Papp = (dc/dt)/(A.Co) where dc/dt = transport rate, mol.s^{-1}. A = surface area of membrane, cm^2. Co = initial concentration in donor chamber, mol/ cm^3.

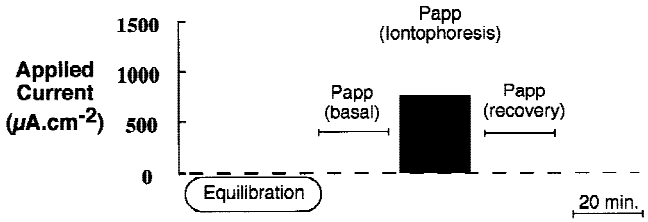


Fig. 1. Schematic illustration of the experimental design employed in these studies. Following an equilibration period, Papp was determined over three consecutive intervals. Iontophoresis was performed by applying a current of 14.3 μEq.cm^{-2} (1.2 mA/cm^2) for 20 minutes. Positive SCC values indicate apical-side negative with respect to the basolateral compartment. The amount of charge transfer was calculated by integration of current and time.

Measurements of transepithelial electrical resistance (TEER) and short circuit current (Isc) were made using Ag/AgCl voltage and current electrodes connected via bridges containing 3M KCl agar gel. These electrodes were attached to the voltage clamp, which was in turn linked to a Maclab® data acquisition system. The voltage was clamped intermittently at 1mV and the corresponding deflection in short circuit current used to calculate TEER by applying the ohmic relationship (R = V/I).

The effect of current on the absorptive flux of the hypothalamic peptide TRH (MW = 362) across Caco-2 monolayer was investigated. 7.5 μCi of ^{3}H -TRH was added apically and measurements were calculated as described above. Similarly, 10 μCi of ^{3}H -dexamethasone (MW = 392.5) was added apically and its absorptive flux was studied as above by radioisotope activity.

In the FITC-Dextran studies, 5mg/ml FITC-Dextran was added apically in all experiments and allowed to equilibrate for approximately 30 minutes before the flux sampling was begun. Samples taken were measured on a LS 50B luminescence spectrometer at an excitation wavelength of 494nm and an emission wavelength of 520 nm. The Papp was measured for each sampling period as outlined before using fluorescence as a probe.

The unpaired Student's t-test was used for statistical analysis with $p < 0.05$ taken as the criterion for significance.

Transmission Electron Microscopy

TEM of the Caco-2 monolayer was used to visualise any intracellular effects. Cells were allowed to equilibrate for approximately 20 minutes in the diffusion chamber prior to the application of current that would transfer a charge of 14.3 μ-Eq.cm^{-2} (1.2 mA/cm^2) across the cell monolayer as in iontophoretic protocol. Cells were immediately fixed in 3% glutaraldehyde and then fixed in osmium tetroxide. To examine

recovery, cells were incubated for 40 minutes in the bathing solution before fixation.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was used to visualise the iontophoretic transport route of FITC-Dextran (MW = 20 kDa) across a Caco-2 monolayer. 14.3 μ-Eq.cm^{-2} (1.2 mA/cm^2) of current was applied across a monolayer of Caco-2 cells grown on Transwell Clear filters (Costar, 6.5mm diameter, 0.4μm pore size). Cells were counterstained with 0.5 μg/ml Texas Red DHPE in the dark for 10 minutes at 37°C. The monolayer was then washed in a solution of 80mM K PIPES, 5mM EGTA, 2mM $MgCl_2$, pH 6.8, followed by a 5 minute incubation (with gentle shaking) in a solution of 80 mM K PIPES, 5mM EGTA, 2mM $MgCl_2$ and 3% paraformaldehyde, pH 6.8. This solution was removed and the filter was incubated for a further 10 minutes (with gentle shaking) in a solution of 100mM NaB_4O_7 and 3% paraformaldehyde, pH 11. After repeated washing in PBS, pH 7.5, filters were incubated for 15 minutes in a 1 mg/ml solution of $NaBH_4$ dissolved in PBS, pH 8. Filters were then washed before finally being mounted on a slide for viewing by confocal laser scanning microscopy.

RESULTS

Permeability Experiments in Caco-2 Cells

The Papp values (cm.s^{-1}) measured for mannitol absorption (apical-basolateral) across a Caco-2 monolayer *in vitro* were constant throughout the entire flux experiment. For example, Papp values determined under open-circuit conditions over two consecutive 40 minute intervals were $0.2 \pm 0.1 \times 10^{-6}$ cm.s^{-1} and $0.3 \pm 0.3 \times 10^{-6}$ cm.s^{-1}; n = 3. Electrophysiological parameters were also stable in these control experiments. Transepithelial potential difference was 1.1 ± 0.1 mV at the beginning and 0.8 ± 0.2 mV at the end of these experiments (n = 3). Similarly, TEER values were 430 ± 12 Ω.cm^2 at the beginning and 376 ± 58 Ω.cm^2 at the end of these experiments (n = 3). Following the application of the test current (14.3 μ-Eq.cm^{-2}) mannitol flux was significantly enhanced by approximately 8.5 fold (Figure 2a and Table 1). Consequent to the termination of the applied current, the Papp decreased to the normal range. Papp values for mannitol absorption before and after application of 14.3 μ-Eq.cm^{-2} were $6.2 \pm 2.7 \times 10^{-7}$ cm.s^{-1} and $6.5 \pm 4.0 \times 10^{-7}$ cm.s^{-1} respectively (n = 4).

The effect of iontophoresis on TRH enhancement across the Caco-2 cell monolayer is outlined in Figure 2a. The Papp for basal TRH absorption was $2.3 \pm 0.1 \times 10^{-6}$ cm.s^{-1} which was significantly increased during application of charge (14.3 μ-Eq.cm^{-2}) within the sampling period ($7.5 \pm 1.1 \times 10^{-6}$ cm.s^{-1}). The 3.3 fold increase in permeability flux of TRH due to the application of current was restored to basal values during the recovery period ($2.8 \pm 0.4 \times 10^{-6}$ cm.s^{-1}) (n = 3).

The absorptive flux of the hydrophobic dexamethasone was significantly enhanced following the application of 14.3 μ-Eq. cm^{-2} (Figure 2a). Papp for dexamethasone absorption was increased from $9.4 \pm 0.9 \times 10^{-6}$ cm.s^{-1} to $14.0 \pm 0.6 \times 10^{-6}$ cm.s^{-1} as a consequence of the current application. The increase in permeability flux was restored to basal values during the recovery period ($6.7 \pm 0.6 \times 10^{-6}$ cm.s^{-1}) (n = 4).

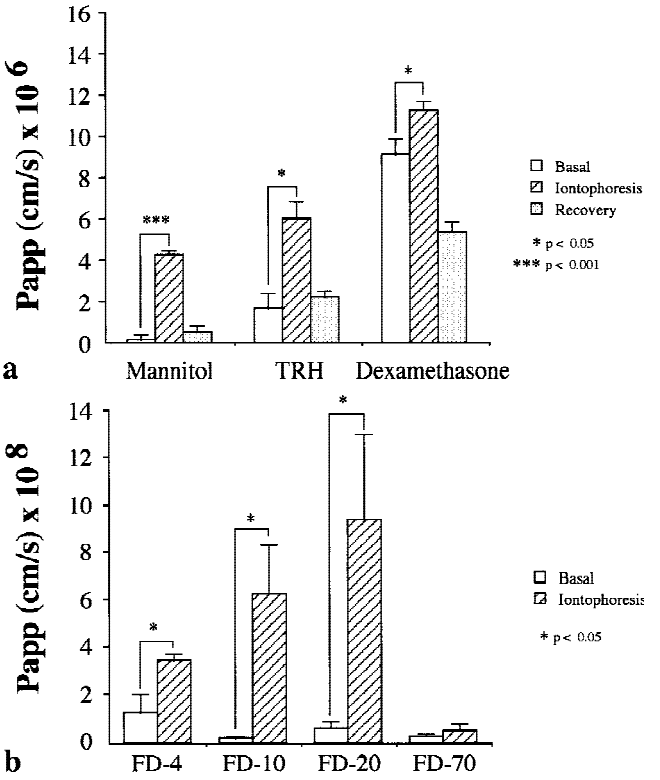


Fig. 2. The effect of iontophoretic stimulus (applied charge) and permeability to various compounds. (a) To mannitol, TRH and dexamethasone; (b) to FD-4, FD-10, FD-20 and FD-70. Permeability was significantly enhanced in response to the application of 14.3 μEq.cm^{-2} (1.2 mA/cm^2) in all instances except for FD-70 ($p < 0.05$).

Table 1b shows the permeability co-efficients of FD-4, FD-10, FD-20 and FD-70 with/without the application of current (14.3 μ-Eq. cm^{-2}). A significant increase in flux was ob-

Table 1a. Permeabillity Coefficients for Mannitol, TRH and Dexamethasone During Passage of Charge Across Caco-2 Monolayer *In Vitro*

	Open Circuit	Iontophoresis
Mannitol Papp (cm · s^{-1}) × 10^6	0.2 ± 0.3 (3)	5.3 ± 0.3^b (4)
TRH Papp (cm · s^{-1}) × 10^6	2.1 ± 0.9 (3)	7.5 ± 1.1^a (3)
Dexamethasone Papp (cm · s^{-1}) × 10^6	11.4 ± 0.9 (4)	14.0 ± 0.5^a (4)

Table 1b. Permeability Coefficients for FD-4, FD-10, FD-20 and FD-70 During Passage of Charge Across Caco-2 Monolayer *In Vitro*

	Open Circuit	Iontophoresis
FD-4 Papp (cm · s^{-1}) × 10^8	1.3 ± 0.8 (5)	3.5 ± 0.3^a (4)
FD-10 Papp (cm · s^{-1}) × 10^8	0.2 ± 0.1 (4)	6.3 ± 2.1^a (4)
FD-20 Papp (cm · s^{-1}) × 10^8	0.6 ± 0.3 (4)	9.5 ± 3.6^a (4)
FD-70 Papp (cm · s^{-1}) × 10^8	0.3 ± 0.1 (3)	0.5 ± 0.3 (3)

Note: ^{a} = $p < 0.05$, ^{b} = $p < 0.0001$ when compared with values obtained from 'open circuit' experiiments. Numbers of tissues used in each group is given in parenthesis.

Table 2. Transepithelial Electrical Resistance Values (A) and Potential Difference Values (B) Were Not Significantly Altered During Passage of Iontophoretic Current

	Open Circuit (n = 10)	Iontophoresis (n = 11)
A. TEER ($\Omega \cdot cm^2$)		
Pre-iontophoresis (Basal)	289 ± 59	315 ± 60
During iontophoresis	298 ± 67	187 ± 49
Post iontophoresis (Recovery)	271 ± 57	326 ± 63
B. Potential difference (mV)		
Pre-iontophoresis (Basal)	0.4 ± 0.2	0.4 ± 0.1
Post iontophoresis (Recovery)	0.4 ± 0.2	0.4 ± 0.1

served for the 4, 10 and 20 kDa dextrans in the iontophoresis model but not for the 70kDa dextran (Figure 2b). There was a 3-, 9-, and 30 fold increase in flux observed for the 4, 10 and 20kDA dextrans respectively.

Potential difference and TEER values were stable at the beginning and end of each experiment (Table 2). There was no statistically significant effect on the integrity of the monolayer as indicated from the TEER values recorded although resistance values tended to decrease during iontophoresis. (Table 2a). A transmembrane potential drop of 0.22 ± 0.06 V and a power dissipation of 0.27 ± 0.07 $mW.cm^{-2}$ was calculated during the iontophoretic treatment.

Transmission Electron Microscopy

Figures 3a-3d are electron micrographs taken of a control Caco-2 monolayer and a monolayer subjected to 14.3

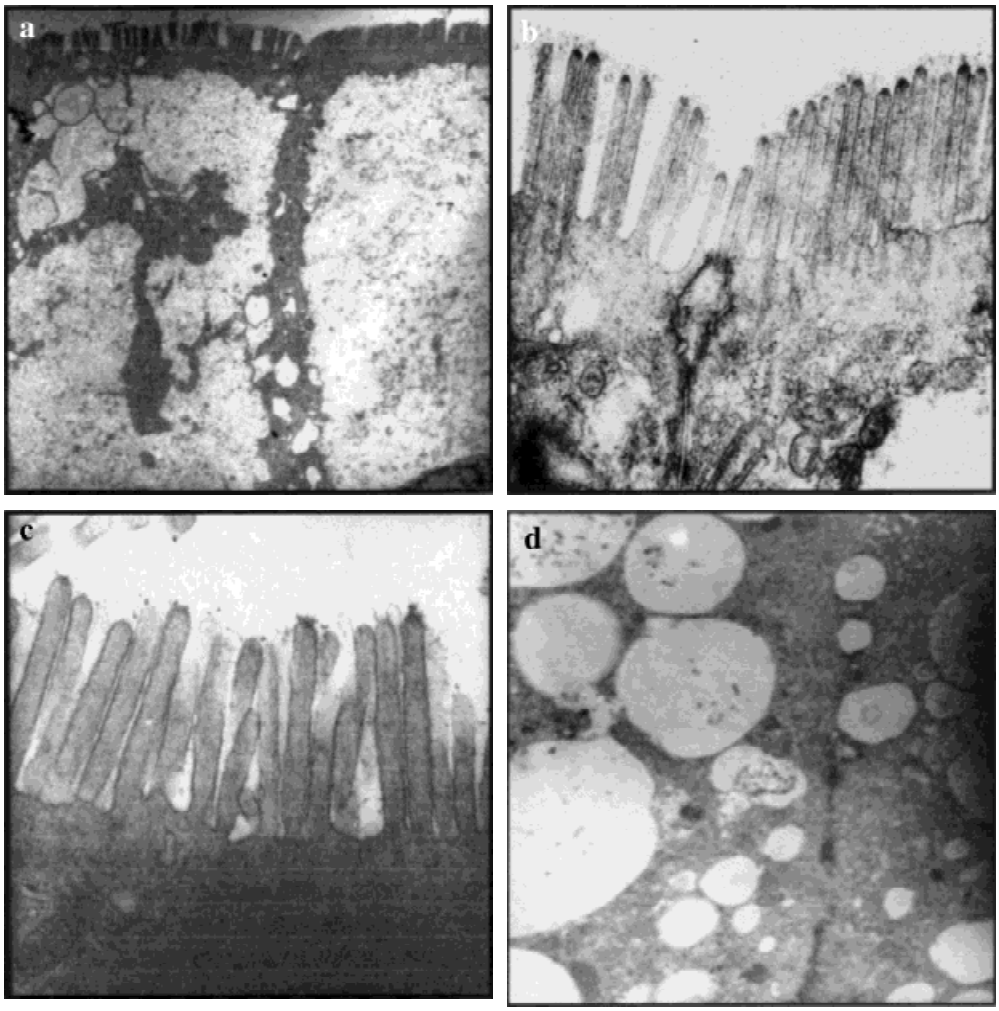


Fig. 3. Electron micrographs of control Caco-2 monolayers and Caco-2 monolayers subjected to 14.3 $\mu Eq.cm^{-2}$ (1.2 mA/cm^2). **(3a)** Electron micrograph of a control Caco-2 monolayer (Mag. X 7k.); **(3b)** Electron micrograph of a control Caco-2 monolayer (Mag. X 20k.); **(3c)** Electron micrograph of a Caco-2 monolayer subjected to 14. 3 $\mu Eq.cm^{-2}$ (1.2 mA/cm^2). Microvilli appear unaffected by the application of current (Mag. X 60k.); **(3d)** Electron micrograph of a Caco-2 monolayer subjected to 14. 3 $\mu Eq.cm^{-2}$ (1.2 mA/cm^2). Tight junctions were highly dilated. Numerous vacuoles have appeared in the cytoplasm (Mag. X 20k.); **(3e)** Electron micrograph of a Caco-2 monolayer subjected to 14. 3 $\mu Eq.cm^{-2}$ (1.2 mA/cm^2) (lumen cathode). Tight junctions were not dilated. Appearance of vacuoles in the cytoplasm was not to the same degree (Mag. X 60k.); **(3f)** Electron micrograph of a Caco-2 monolayer fixed 40 minutes after current application. Vacuolous effect has reduced (Mag. X 20k.); **(3g)** Electron micrograph of a Caco-2 monolayer fixed 40 minutes after current application. Tight junctions appear widened but not as dilated (Mag. X 60k.).

μ-Eq.cm^{-2} charge (test current used in permeability experiments above).

Microvilli were normal in control (Figure 3b) and test samples (Figure 3c) which indicates no direct insult/toxic effect to the monolayer as a result of the applied current. Tight junctions can be seen to have been dilated in the test specimen (Figure 3d). The main point of interest from the test electron micrograph (Fig. 3d) was the appearance of large vacuoles in the cytoplasm. These vacuoles appeared to be organised and may be a consequence of the applied current. Figure 3e is an EM of a Caco-2 monolayer subjected to current (14.3 μ-Eq.cm^{-2}) applied in the basolateral-to-apical direction. It is clear from this that the effect on the epithelia was not similar to that seen when the electric field was applied in the apical-to-basolateral direction; the dilation of the tight junction was not present and the high degree of vacuoles appearing was not as prevalent as the 'apical-to-basolateral' test specimen. Both apical and basolateral membranes showed no signs of damage/toxic insult. Figures 3f-3g are EMs of Caco-2 monolayers subjected to current and allowed to recover for 40 minutes before fixation with 3% glutaraldehyde. The vacuolous effect was reduced markedly compared to the test monolayer seen in Figure 2b. The tight-junctions also appear to be restored to their characteristic shape.

Confocal Laser Scanning Microscopy

Figure 4a-4d is a series of four images of a Caco-2 monolayer under a confocal laser scanning microscope. The horizontal images move from the apical surface of the monolayer down into the cell in approximately 2.5 μm steps towards the basolateral membrane on the filter. The counterstain Texas Red DHPE was localised in the intercellular spaces of the mosaic-like Caco-2 cell monolayer. Slight traces of the green fluorescing probe FD-20 were observed. Figures 4e-4h represent a similar series of images where conditions were identical but for the application of current. There was a considerable degree of fluorescent probe located in the intercellular spaces and it can be traced from the apical side right the way through to the basolateral side. The FITC-labelling did not appear to be uniformly distributed but this was not quantitatively assessed.

DISCUSSION

The passive permeability of various drugs across the Caco-2 model correlates with the percentage dose absorbed in humans (5). Permeability profiles comparing human colon, rat colon and the Caco-2 cells show a good correlation and can be used to predict *in vivo* absorption (13). The Caco-2 cell

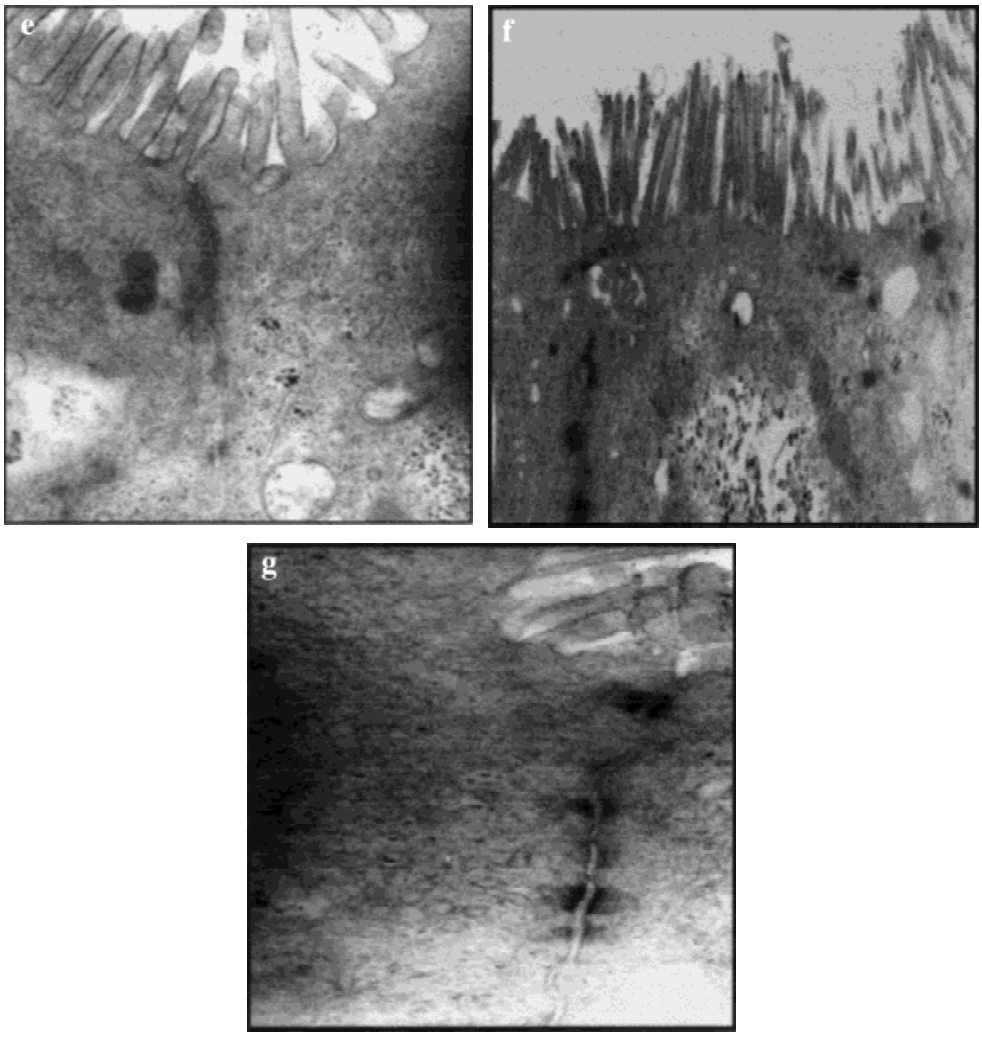
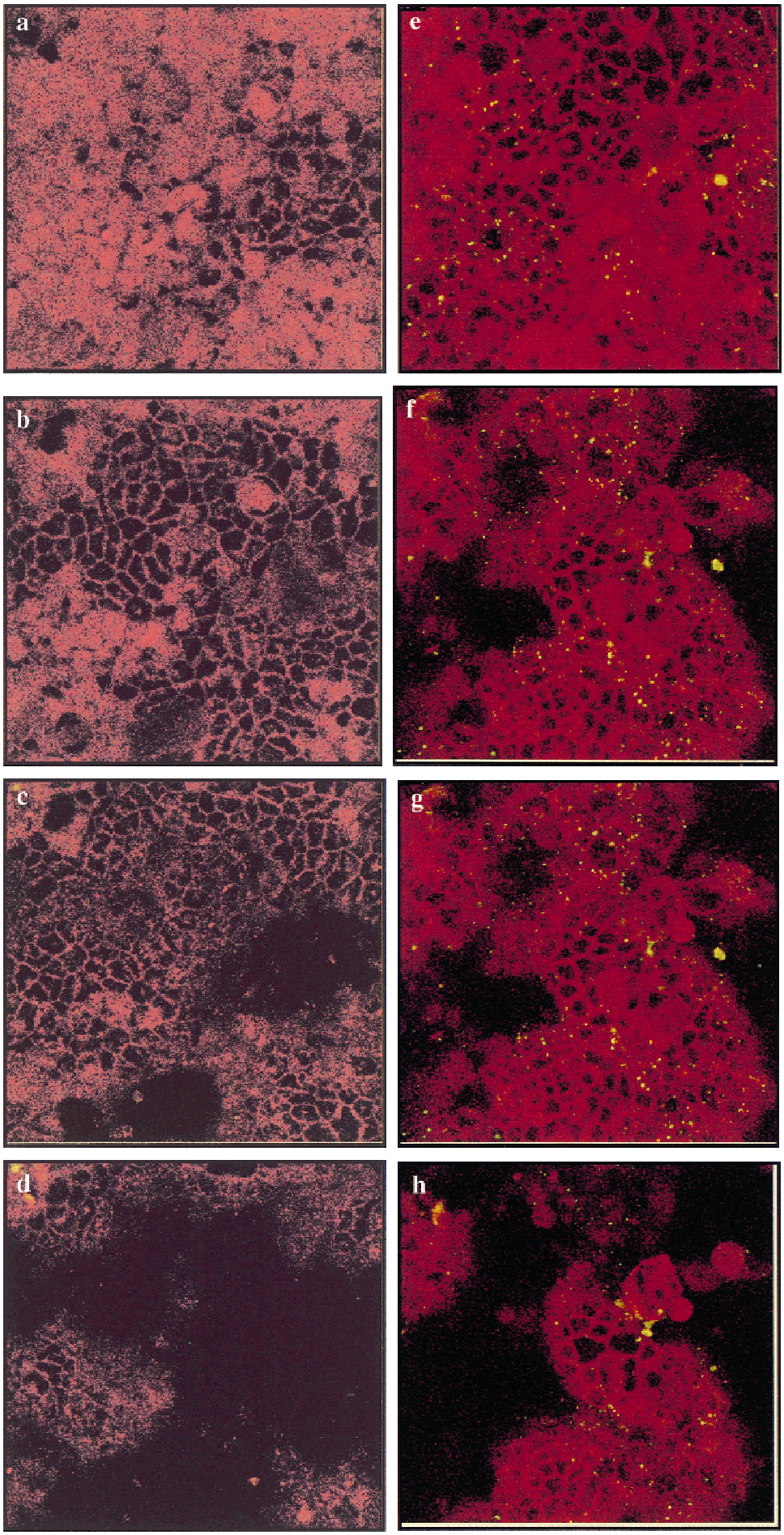


Fig. 3. (*Continued*)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line would appear to be a suitable choice as a model in studying the effect of an applied current on drug absorption in humans.

The establishment of an electrical potential gradient across an epithelial monolayer can be expected to drive cations from the anode and anions from the cathode as a result of electrostatic repulsion (14), while a neutral polar compound such as mannitol does not undergo flux enhancement by electrorepulsion but rather as a direct influence of the electro-osmotic effect on the convective solvent flow that occurs during iontophoresis (15). This has been demonstrated for rat colon (12) and now for the Caco-2 cell monolayer in respect of mannitol and TRH. The enhanced mannitol flux across Caco-2 cell monolayers indicates that the Caco-2 cell line is a suitable model for this application. Transport across the cells was increased 8.5 fold, whereas mannitol flux across rat colonic tissue was found to be increased 2.5 fold. The application of 14.3 μ-Eq.cm^{-2} of charge caused a 3.3 fold increase of TRH across the Caco-2 cell monolayer in comparison to a 2-fold increase across rat colon. This greater increase in permeability can be accounted for by the relatively tighter junctions of the Caco-2 monolayer. The high resistance values of the Caco-2 monolayer means that the transport of hydrophilic compounds was reduced to lower levels than that of the rat colonic epithelial tissue and so small changes in paracellular permeability are more readily distinguishable (13). These results indicate that the Caco-2 model is a more sensitive model for investigating iontophoresis of drugs across the colon, perhaps because Caco-2 tight junctions can be opened more readily and are therefore more discriminating in drug absorption (5).

The iontophoresis of dexamethasone was investigated to compare a hydrophobic molecule to a hydrophilic one i.e. mannitol. The lipophilic dexamethasone is absorbed across the cell membrane and crosses from the lumen to the serosal side of the epithelium via the transcellular route (5). This is reflected in its high Papp of 1×10^{-5} cm. s^{-1} (mannitol had a Papp of 0.2×10^{-6} cm. s^{-1}). The transfer of dexamethasone was slightly increased by the application of current. This does not however suggest that the iontophoretic current pathway was transcellular. It is reasonable to expect a small proportion of a small such as dexamethasone molecule (MW = 392.5) is being transported paracellularly by the iontophoresis. The results with dexamethasone demonstrate that the absorptive flux of both hydrophilic and hydrophobic molecules can be increased significantly.

Being hydrophilic, the fluorescein isothiocyanate (FITC)-labelled dextrans move through the paracellular space (16). The fluorescently-labelled dextrans (4, 10, 20, and 70 kDa) were used to investigate the size exclusion properties of iontophoresis in the Caco-2 monolayers. The experiments define the size limitation to be less than 70 kDa. As a direct effect of current being applied, an increase in flux was observed for the 4, 10, and 20 kDa dextrans and appears to proportional to molecular weight. Being a neutrally charged molecule, dextran is transported across the epithelium primarily due to convective flow. The effect of convective flow was therefore greater as the size of the molecule increases, a finding supported by iontophoretic experiments with skin (17). These results suggest that electro-osmosis will play a decisive role in peptide/macromolecule iontophoresis.

Similar to the results reported for rat colon there was an observed reduction in TEER recorded after the application of current accompanied by a significant rise in permeability, suggesting that the iontophoretic pathway was indeed the paracellular route. This was supported by a return to normal resistance levels, paralleled by the restoration of basal flux values. This was further supported by transmission electron micrographs taken of Caco-2 cells post-iontophoresis; tight junction dilation was apparent suggesting that iontophoresis was increasing the transport of drugs across the epithelial cells via the paracellular route. The TEMs demonstrate the applied test current was not causing damage/toxic insult to the cells, and this was consistent with the stable electrical parameters within the normal range at the end of the experiment**.** Similar histological changes have been seen when absorptive intestinal cells were exposed to glucose. Transient appearance of vacuoles, accompanied with decreased membrane resistance and greater permeability were observed (18). Although the mechanisms involved in such changes remain unclear, no cellular damage was inferred. The recovery TEMs of the monolayer highlight that the effect on the tight junctions was reversible. Finally, further supporting evidence of the involvement of the paracellular pathway as the iontophoretic transport route was provided by confocal microscopy. Visual tracing was seen for a fluorescent probe from the apical side right through to the basolateral membrane under iontophoretic conditions. FITC label was not apparent within cells but appeared to be co-localized with junctional spaces (stained with Texas Red) at all levels of the monolayer, as probed with confocal microscopy. The distribution of FITC label appeared to be non-uniform. We do not know why this may be but is unlikely to be accounted for by localized membrane damage since the Texas Red distribution is not consistent with this and, secondly, there was no evidence of damage to the monolayers as discussed above. This suggests that the iontophoretic current was increasing the transport of drugs across the epithelial cells via the paracellular pathway.

In conclusion it appears that electrical manipulation of the paracellular pathway i.e. controlled reversible opening of the junctional complex by iontophoresis, can be used to increase the absorption of small peptides in human *in vitro* intestinal monolayers. This data furthers the initial research in iontophoresis made for rat intestinal tissue segments (12). The next steps will be to assess if such an effect can be achieved in an *in vivo* system such as rat intestinal loops.

ACKNOWLEDGMENTS

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Fig. 4. (*opposite*) Confocal images of a Caco-2 monolayers at successive depths from apical surface towards basolateral membrane. **(a-d),** 5 mg/ml FITC-Dextran (20kDa) was added apically for 30 minutes before staining. Texas Red DHPE counterstaining was localized in the intercellular spaces. Slight traces of FD-20 (yellow staining) can be distinguished; **(e-h),** 5 mg/ml FITC-Dextran (20kDa) was added apically and 14.3 μ-Eq. cm^2 (1.2 mA/cm^2) charge applied across monolayer for 30 minutes before staining. Texas Red DHPE counterstaining was localized in the intercellular spaces. Large quantities of FD-20 (yellow staining) were detected in the intercellular spaces running from the apical to the basolateral side.

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