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Polycation-induced enhancement of epithelial paracellular permeability is independent of tight junctional characteristics

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The nature of polycation-induced change in transepithelial permeability was investigated in strains I (*tight*) and II (*leaky*) MDCK epithelial monolayers. Apical exposure to poly(L-lysine) (PLL, mol. wt. (MW) ≈ 20 000) induced a dose-dependent increase in transepithelial conductance (G_T) in both strains which correlated with increasing transepithelial flux of extracellular markers (thiourea/inulin) indicating that PLL enhanced paracellular permeability in these epithelia. Coincident with the increase in G_T, PLL also induced an inward short circuit current (I_{sc}) which was associated with the early phase of the increase in G_T and may be responsible for part of it. Morphological studies showed that immunofluorescent staining of the tight junction protein, ZO-1, was abolished following PLL exposure. In addition, F-actin staining in monolayers challenged with PLL demonstrated breaks in the *zonulae occludentes* at the apical surface. PLL had similar effects on monolayers of T_{84} and HCT-8 human intestinal cells indicating that polycation action may be general for a range of epithelial types. We conclude that epithelial exposure to polycations results in opening of the paracellular route by mechanisms which are independent of tight junction characteristics.

Introduction

Polycationic macromolecules have diverse effects upon epithelial permeability in a variety of tissues [1–5]. In *Necturus* gallbladder, exposure to the polycation, protamine, resulted in an increased transepithelial resistance (R_T) [2,5]. Apical membrane resistance (R_a) was also increased due to a reduced apical K^+ conductance [5]. The increase in R_T was associated with a concomitant reduction in paracellular cation selectivity which was correlated with alterations in tight junction structure [1]. The protamine-dependent increase in transepithelial resistance has been used to define paracellular contributions to total conductance of renal descending thin limb (TDL; another 'leaky' epithelium) [3]. However, in a tight epithelium composed of cultured strain I Madin-Darby canine kidney (MDCK) cell [4], protamine exposure resulted in a marked fall in R_T. This was accompanied by an increased transepithelial flux of extracellular markers, consistent with a polycation-dependent increase in paracellular permeability.

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The present investigation had two main objectives. Firstly, to investigate the generality of the finding that polycations can induce substantial change in paracellular permeability and that these changes are dependent upon the nature of the junctional complex. To this end we have utilised two distinct strains of MDCK cells: strain I, which display characteristics consistent with those of 'tight' epithelia ($R_T \approx 1.5$–4 kΩ cm^2) and strain II which have 'leaky' cation selective tight junctions with R_T values of typically 100–200 Ω cm^2 [6]. In addition two human colonic epithelial cell types which form 'tight' epithelia were used; the human intestinal adenocarcinoma cell-lines T_{84} and HCT-8. Secondly, to determine whether any changes in paracellular marker fluxes are accompanied by structural changes in the tight junction complex.

Materials and Methods

Cell culture

MDCK cells of both Strain I (passages 69–76) and Strain II (passages 113–118) [6] were cultured in Eagle's minimum essential medium (EMEM) containing Earle's salts supplemented with foetal calf serum (2% vol/vol), horse serum (8% vol/vol), L-glutamine (4 mM) and kanamycin (100 μg/ml). T_{84} cells [7] (passages 64–70) were cultured in a mixture of Dulbecco's minimum

essential medium (glucose 1.5 g/l) and Ham's F12 medium supplemented with newborn calf serum (5%) and penicillin/streptomycin (20 U/ml). HCT-8 cells [8] were cultured in RPMI medium containing horse serum (10%), sodium pyruvate (2 mM), L-glutamine (4 mM) and penicillin/streptomycin (20 U/ml). Cell lines were passaged with 0.05% trypsin/0.5 mM EDTA. Both Strain I and II MDCK cells were treated identically.

For experimental purposes, cells were seeded at high density (10^6 cells/ml suspension) into tissue culture inserts incorporating a nitrocellulose filter support (Millicell HA, Millipore) or an inorganic membrane tissue culture support (Anotec, Nunc, Denmark). For T_{84} and HCT-8 cells the filters were precoated with collagen to provide a suitable growth matrix. No pretreatment was necessary for the two strains of MDCK cells.

Electrophysiological measurements

Epithelial layers (Millicell HA filter supports) were clamped between the two halves of a specially modified Ussing chamber. The bathing medium was a modified Krebs buffer [6] and had the following composition (mmol/l) NaCl 137; KCl 5.4; $CaCl_2$ 2.8; $MgSO_4$ 1.2; NaH_2PO_4 0.3; KH_2PO_4 0.4; HCl 12; Tris base 14; glucose 10; pH 7.40. The chamber was water jacketed to maintain the bathing buffer at 37°C throughout the experiment. The transepithelial potential difference (V_T) was measured by two Calomel electrodes connected to each half of the chamber by 3 M KCl/3% agar bridges. Two current passing Ag/AgCl electrodes were similarly connected to each half of the chamber. The Calomel half cells and the Ag/AgCl electrodes were coupled to a voltage/current clamp (DVC-1000; World Precision Instruments). Short circuit current (I_{sc}) was determined by measuring the current required to clamp V_T at 0 mV. The transepithelial electrical conductance across the monolayers (G_T) was determined from the PD measured in response to regularly imposed current pulses (20 μA, 1 s duration every 5 s) generated by the voltage/current clamp. The voltage and current output signals from the clamp unit were recorded on a two channel paper chart recorder (Vitatron, UK). In addition, G_T measurements were made using a combined voltmeter and constant current source (Evometer, World Precision Instruments) connected to the external bathing medium and to the insert interior by Ag/AgCl electrodes. Similar measurements of G_T were obtained using the two techniques.

Measurement of biionic and dilution potential differences

The paracellular pathway in strain II MDCK epithelium is cation selective [9]. Selectivity was determined by either (1) measurement of biionic electrical potential differences (choline:Na^+) achieved by isosmotic replacement of NaCl in the basolateral bathing medium with choline chloride or (2) measurement of dilution potential differences after basolateral NaCl was replaced by isosmotic mannitol. The selectivity of the paracellular pathway for Na^+ over Cl^- (β) was calculated from dilution potential difference determinates using a modification of the Goldman-Hodgkin-Katz equation:

$$\Phi_{ms} = \frac{RT}{F} \ln \frac{\alpha[Na]_o + \beta[Cl]_i}{\alpha[Na]_i + \beta[Cl]_o}$$

where Φ_{ms} is the dilution potential difference (mV), α is the ionic activity coefficient (assume $\alpha = 1$), β is the ratio of Cl^- permeability to Na^+ permeability (P_{Cl}/P_{Na}), $[x]_o$ and $[x]_i$ denote the respective ion concentrations in the apical and basolateral bathing solutions and R, T and F have their usual meanings.

Measurement of extracellular marker fluxes

MDCK epithelial layers on Anocell tissue culture inserts were placed into 6-well tissue culture plates with 2 ml Krebs buffer (see above for composition) in each well. The plates were placed on a bench incubator (maintaining buffer temperature at 37°C) and either Krebs buffer alone or buffer containing poly(L-lysine) was added to the apical surface of the monolayers for 5 min. The inserts were then emptied and filled with 0.5 ml Krebs buffer containing 37 kBq/ml [^{14}C]thiourea (MW = 76) and/or 37 kBq/ml [^{3}H]inulin/[^{14}C]inulin (MW $\approx$ 5000). These molecules are normally confined to the extracellular domain and therefore any appearance on the contralateral side should indicate transport via the paracellular pathway. An initial 200-μl sample was taken from the apical bathing solution at the onset of the experiment and subsequent 200-μl samples were removed from the basolateral bathing solution every sixty min (this volume was replaced by an equal volume of fresh buffer). The radiolabelled thiourea and inulin were counted in a liquid scintillation counter (Beckman LS 5000CE) and the apical to basolateral fluxes ($J_{a\text{-}b}$) for both markers were calculated from the following equation:

$$J_{a\text{-}b} = \frac{(C \cdot V)/t}{(H/S)A}$$

where C is the accumulated activity calculated for each basolateral sample (cpm), V is the volume of the basolateral bathing medium (ml), t is time (min), H is the activity of the apical bathing medium (cpm/ml), S is the solution specific activity for the apical bathing medium (nmol/ml) and A is the effective growth area

of the tissue culture inserts (cm^2). Transepithelial conductance was measured continually as described above.

Morphological studies

ZO-1 localisation. For immunofluorescence studies, MDCK cells were grown on inorganic filter supports. Following apical exposure to PLL (MW ≈ 20000; 50 μg/ml) for 10 min, the cells were fixed and permeabilised in methanol at −20°C for 30 min and were then rehydrated in phosphate buffered saline (PBS). Hybridoma tissue culture fluid supernatants containing ZO-1 antibody, R26.4C [10] were used undiluted and applied to both apical and basolateral surfaces (300 μl each side) for one h at room temperature. Following this, the filters were washed three times in PBS and incubated for one h at room temperature with FITC-labelled goat anti-mouse antibody (1:50 dilution in PBS). Following a final wash in PBS, the filters were mounted onto glass slides with a commercial antifading agent (Vectashield, Vector Laboratories). Slides were examined using a confocal laser scanning microscope (Bio-Rad MRC 600) attached to a Nikon inverted microscope.

F-actin localisation. Cells were stained with TRITC-phalloidin using the method described by Peterson and Gruenhaupt (1990) [4]. Following apical exposure to PLL (see above), monolayers were washed three times in PBS and fixed in formaldehyde (3.7% in PBS) for 10 min at 25°C. The cells were then permeabilised with Triton X-100 (0.1% in PBS) for 10 min at 25°C and washed three times in PBS. The monolayers were then incubated with TRITC-labelled phalloidin for 60 min at 37°C in the dark. The filters were mounted onto glass slides and examined by confocal microscopy (see above).

Materials

Poly(L-lysine), poly(D-lysine), poly(L-arginine), poly (L-ornithine), poly(L-histidine), polyglycine, poly(L-leucine), poly(L-aspartic acid), poly(L-glutamic acid), poly(Lys,Ala), protamine, TRITC-phalloidin and FITC-labelled goat anti-mouse IgG were supplied by Sigma (Poole, Dorset). ZO-1 hybridoma cells (R26.4C) were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. All other chemicals were of Analar grade and were supplied by Merck (Poole, Dorset).

Statistics

Results are expressed as means ± S.E. for all experiments. Statistical significance was calculated from Student's *t*-test by nonparametric analysis (Mann-Whitney), where appropriate.

Results

Effect of PLL on I_{sc} and G_T in strain I MDCK epithelial monolayers

Addition of PLL (MW ≈ 20000; 50 μg/ml) to the apical bathing solution of strain I MDCK monolayers resulted in a transient increase in inward I_{sc} from a low resting level (Fig. 1), reaching a maximum value of 85.5 ± 8.7 (6) μA cm^{-2} after 90 s and returning to resting values within 5 min. This pattern of response was typical of the majority of experiments and was totally consistent within a single batch of monolayers. However, the magnitude of the response varied from batch to batch. The increased I_{sc} was always accompanied by a simultaneous and substantial increase in G_T (Fig. 1). In the series of experiments presented in Fig. 1, G_T increased from 0.91 ± 0.08 mS cm^{-2} (6) to

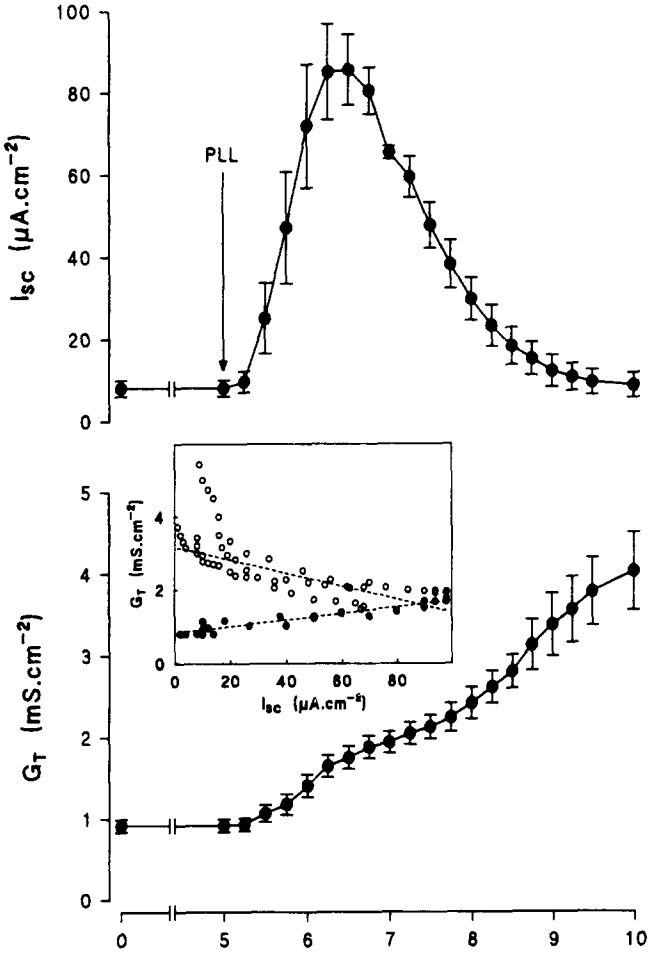

Fig. 1. Effect of PLL on I_{sc} and G_T in one batch of strain I MDCK epithelial cell monolayers. PLL (50 μg/ml) was added to the apical bathing medium after 5 min. Values are expressed as means ± S.E. for six experiments, one observation per experiment at each time point. (Inset) Plot of I_{sc} against G_T derived from Figs. 1a and b. Linear regression was performed for the rising (•) and falling (○) phases of I_{sc} response. See text for details.

4.02 ± 0.48 mS cm^{-2} ($P < 0.001$) within 5 min. A similar rise in G_T was observed upon apical exposure to another polycation, protamine (from 0.67 ± 0.08 (5) mS cm^{-2} to 1.25 ± 0.14 (5) mS cm^{-2}; $P < 0.01$). The increased G_T in response to PLL was biphasic in nature, the early rapid phase appeared to be associated with the increase in I_{sc}, whilst G_T then continued to increase as I_{sc} declined.

Yonath and Civan (1971) [11] have suggested a graphical method to estimate the relative contributions of cellular and paracellular conductance to the overall G_T. The inset in Fig. 1 shows a plot of G_T/I_{sc} during exposure to PLL. Two linear components are evident for the rising and falling phases of the I_{sc} response. By extrapolating to zero I_{sc}, the magnitude of the intercept on the G_T axis for the two phases is indicative of the magnitude of paracellular conductance. This suggests that the initial increase in G_T (concurrent with the rising phase of the I_{sc} response) was largely associated with increased cellular conductance whereas the later change was due to increasing paracellular conductance. This was most marked at 8–10 min where G_T continued to increase despite I_{sc} being close to zero, i.e., there was a marked deviation from linearity parallel to the G_T axis.

The change in G_T in response to apical PLL first occurred at a concentration of 5 μg/ml (Fig. 2A), the magnitude of the response increasing with PLL dose up to 100 μg/ml. A similar dose–response relationship was obtained for the effect of apical PLL on I_{sc} (Fig. 2B). Addition of PLL (100 μg/ml) to the basolateral bathing medium had no effect on either G_T or I_{sc} (Fig. 2) even at concentrations in excess of 100 μg/ml (data not shown).

The effects of apically applied PLL upon G_T were found to be irreversible in the short term (up to 2 h)

TABLE I

Effect of various polyamino acids on G_T across strain I MDCK monolayers

Values expressed as means ± S.E. for six experiments. * $P < 0.02$; ** $P < 0.0001$ vs. pre-exposure values.

Compound (50 μg/ml)	Pre-G_T (mS cm^{-2})	Post-G_T (mS cm^{-2})
Control	0.54 ± 0.03	0.61 ± 0.04
Poly(L-lysine)	0.64 ± 0.03	2.83 ± 0.12 **
Poly(D-lysine)	0.45 ± 0.03	2.77 ± 0.19 **
Poly(L-arginine)	0.43 ± 0.08	3.00 ± 0.04 **
Poly(L-ornithine)	0.66 ± 0.04	3.14 ± 0.11 **
Poly(L-histidine)	0.74 ± 0.05	0.91 ± 0.04 *
Polyglycine	0.40 ± 0.04	0.50 ± 0.06
Poly(L-leucine)	0.44 ± 0.06	0.50 ± 0.06
Poly(L-aspartic acid)	0.72 ± 0.07	0.85 ± 0.15
Poly(L-glutamic acid)	0.38 ± 0.24	0.35 ± 0.02
Poly(Lys:Ala) 1:1	0.62 ± 0.04	2.13 ± 0.06 **
Poly(Lys:Ala) 2:1	0.59 ± 0.06	3.12 ± 0.03 **
Poly(Lys:Ala) 3:1	0.58 ± 0.07	3.44 ± 0.07 **

and it was observed that a brief one min exposure (followed by washing) would induce a full response (increased G_T and increased inward I_{sc}) with a similar time course to that shown in Fig. 1.

To investigate the viability of the epithelial monolayers which had been exposed to PLL a series of experiments were undertaken in which adrenaline (10 μM) was added to the basolateral bathing medium after completion of the PLL-induced change in I_{sc}. Adrenaline caused an increased inward I_{sc} (due to electrogenic Cl^- secretion [12]), the magnitude of which was not different from that observed in non PLL-treated cells (Control ΔI_{sc} 9.25 ± 0.48 (4) μA cm^{-2}; PLL ΔI_{sc} 9.29 ± 1.89 (7) μA cm^{-2}; n.s.).

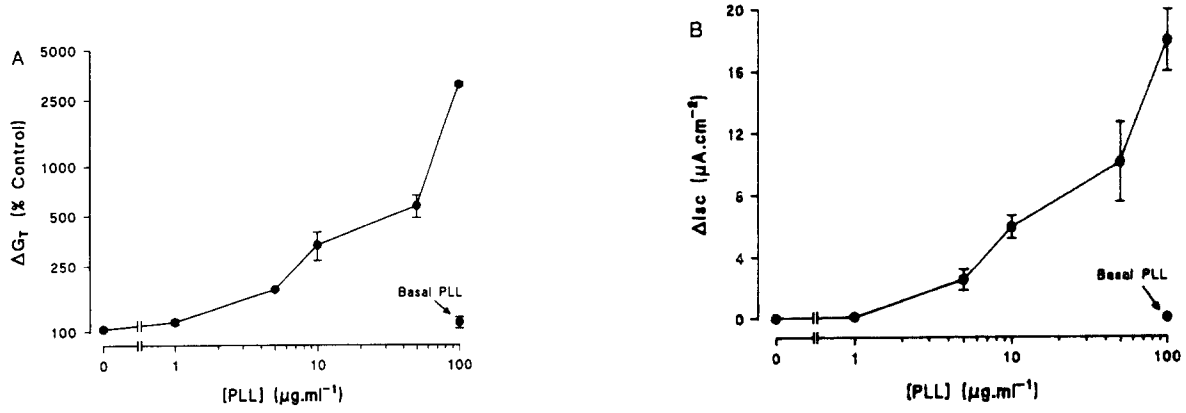

Fig. 2. (A) Dose–response curve for the effect of apically applied PLL on G_T across strain I MDCK epithelial cell monolayers. Values represent the mean G_T (expressed as a percentage of the control $G_T \pm$ S.E.) after 20 min exposure to each PLL concentration for five experiments, one observation per experiment. The effect of a 100 μg/ml dose of PLL applied to the basolateral bathing medium is shown for comparison. (B) Dose–response curve for the effect of apically applied PLL on I_{sc} in strain I MDCK epithelial cell monolayers. Values represent the mean maximum I_{sc} change (± S.E.) at each PLL concentration for five experiments, one observation per experiment. The effect of a 100 μg/ml dose of PLL applied to the basolateral bathing medium is shown for comparison.

Effect of different macromolecules on G_T in strain I MDCK monolayers

The effects of 30 min exposure to a range of different macromolecules (all MW ≈ 20000; 50 μg/ml) on G_T was investigated in strain I MDCK cells (Table I). All polycationic molecules (PLL, poly(D-lysine), poly (L-arginine), poly(L-ornithine), poly(L-histidine)) induced a significant increase in G_T. Poly(L-histidine) had lesser effects than the other polycations tested. Poly(D-lysine) had a similar potency to PLL indicating that D/L isomerism is not an important factor in this case. The polyanionic molecules, poly(L-aspartic acid) and poly(L-glutamic acid) had no significant effect on G_T. Similarly, the neutral species, polyglycine and poly(L-leucine) were without effect.

Table I also shows the effects of random copolymers of lysine and alanine (poly(Lys,Ala)) on strain I MDCK G_T. Three different ratios of lysine:alanine were tested: 1:1, 2:1 and 3:1 (all MW ≈ 20000; 50 μg/ml). All three copolymers significantly ($P < 0.001$) increased G_T, however, the 3:1 molecule had a significantly greater effect than the 2:1 ($P < 0.02$) and 1:1 ($P < 0.001$) molecules.

Effect of PLL on I_{sc}, G_T, Na^+/choline biionic PD and mannitol diffusion PD in strain II MDCK epithelial monolayers

Addition of PLL to the apical bathing solution of strain II MDCK monolayers resulted in the stimulation of a significant inward I_{sc} (Fig. 3a). The pattern of response was similar to that observed with strain I MDCK cells, although the magnitude of the response was considerably lower (I_{sc} at peak = 1.88 ± 0.32 (6); $P < 0.001$). This inward I_{sc} was accompanied by a significant increase in G_T though epithelial conductance continued to increase following the decline in I_{sc} (Fig. 3a) (Control G_T = 8.78 ± 0.95 (6) mS cm^{-2}, PLL G_T (after 5 min exposure) = 33.40 ± 4.01 (6) mS cm^{-2}; $P < 0.001$). Increased epithelial conductance was accompanied by a decrease in the diffusion potential differences developed in response to basolateral NaCl replacement with either choline chloride (CPD) or mannitol (MPD) (Table II). This fall in diffusion PD was the result of a decrease in the selectivity of the paracellular pathway for Na^+ over Cl^- (Table II; β). There was no significant difference between the diffusion PD's generated between choline and mannitol.

These data are, therefore, suggestive of an increased paracellular permeability associated with a decreased junctional selectivity. Similarly, protamine exposure also caused a rise in G_T (from 3.85 ± 0.21 (5) mS cm^{-2} to 7.48 ± 0.60 (5); $P < 0.001$, after 20 min) and reduced the selectivity of the paracellular pathway (CPD was

TABLE II

Effect of poly(L-lysine) (50 μg/ml) mannitol on dilution potential difference (MPD), Na^+:choline biionic potential difference (CPD) and P_{Cl}/P_{Na} (β) in strain II MDCK monolayers

Values expressed as means ± S.E. (n = number of experiments).

	Pre-PLL ($n = 7$)	Post-PLL (10 min) ($n = 5$)	Δ paired values ($n = 5$)
MPD (mV)	22.7 ± 4.3	5.1 ± 2.2	17.5 ± 2.5 $P < 0.001$
CPD (mV)	20.7 ± 3.3	3.8 ± 1.6	17.8 ± 3.0 $P < 0.001$
β (P_{Cl}/P_{Na}) [a]	0.387	0.795	

[a] For definition of β see text.

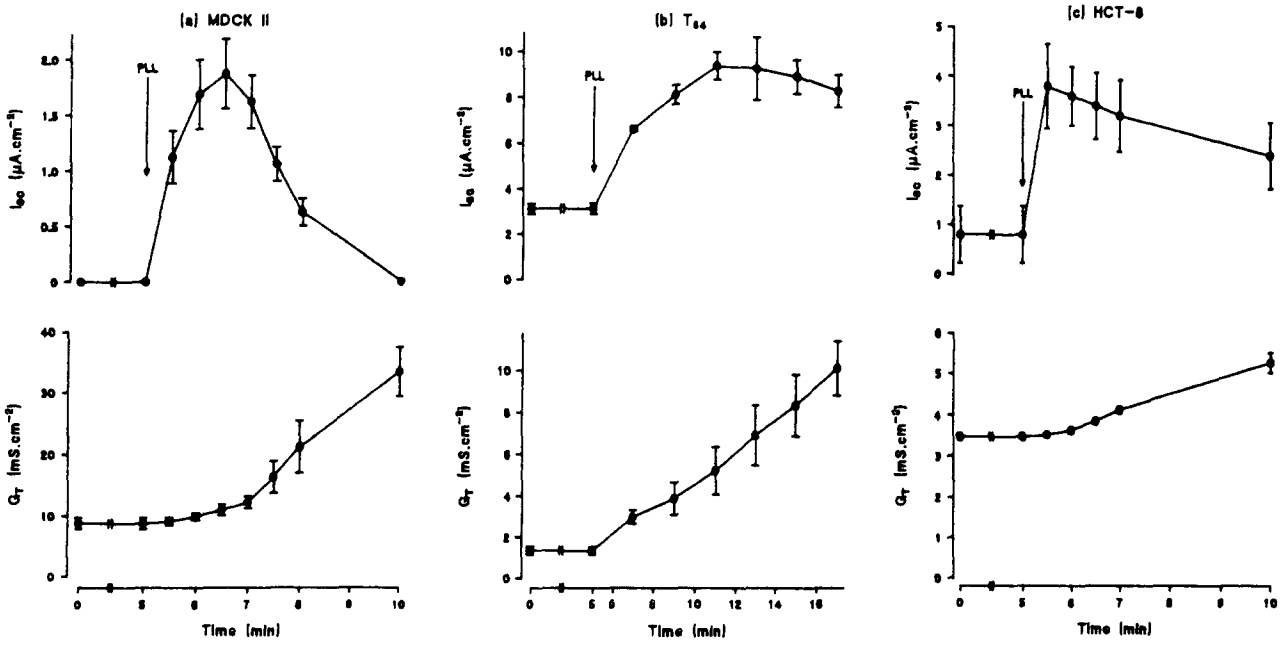

Fig. 3. The effect of PLL on I_{sc} and G_T in (a) MDCK strain II, (b) T_{84} and (c) HCT-8 epithelial cell monolayers. PLL (50 μg/ml) was applied to the apical bathing medium after 5 min. Values represent means ± S.E. for six experiments, one observation per experiment at each time point.

reduced from 23.4 ± 0.8 (5) mV to 5.3 ± 1.2 (5) mV, $P < 0.0001$; MPD was reduced from 35.5 ± 1.2 (5) mV to 23.6 ± 3.8 (5) mV, $P < 0.02$). Thus both PLL and protamine had similar effects in this epithelium.

Effect of PLL on I_{sc} and G_T in human intestinal (T_{84} and HCT-8) epithelial monolayers

T_{84} cells. When PLL (50 μg/ml) was applied apically to T_{84} epithelial monolayers (Fig. 3b) a similar transient increase in I_{sc} to that obtained with MDCK epithelia was observed. The maximum increase in I_{sc} was attained after 5 min exposure when mean values of 9.38 ± 0.62 (6) μA cm^{-2} ($P < 0.001$) were recorded. This increase in I_{sc} was considerably lower than observed with strain I MDCK but was generally sustained over a longer time scale. The increased inward I_{sc} was again accompanied by a substantial rise in G_T (increasing from 1.36 ± 0.10 (6) mS cm^{-2} to 10.14 ± 0.66 (6) mS cm^{-2} ($P < 0.0001$) within 12 min) (Fig. 3b). As with MDCK epithelia, the rise in G_T continued despite a slight fall in I_{sc}.

HCT-8 cells. In similar experiments PLL (50 μg/ml) added to the apical bathing medium of HCT-8 monolayers again resulted in a small transient increase in inward I_{sc} (Fig. 3c) (from 0.81 ± 0.58 (6) μA cm^{-2} to 3.84 ± 0.86 (6) μA cm^{-2} ($P < 0.02$)). PLL exposure also caused an increase in G_T (Fig. 3c) but this was relatively small (from 3.48 ± 0.09 (6) mS cm^{-2} to 5.27 ± 0.25 (6) mS cm^{-2} ($P < 0.001$) after 10 min).

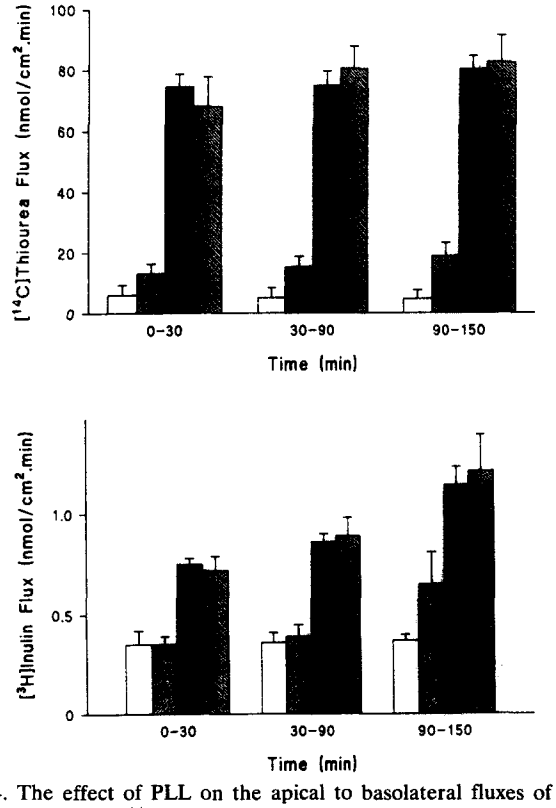

Fig. 4. The effect of PLL on the apical to basolateral fluxes of (a) [^{3}H]inulin and (b) [^{14}C]thiourea across strain I MDCK epithelial cell monolayers. Control layers (open bars) are compared with layers exposed to PLL at either 10 μg/ml (▨), 50 μg/ml (■) or 100 μg/ml (▩). Values are expressed as means ± S.E. for six experiments.

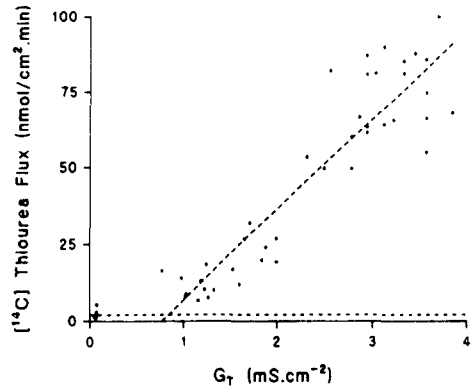

Fig. 5. Plot of [^{14}C]thiourea flux against transepithelial conductance (G_T) in strain I MDCK epithelial cell monolayers. Data from Fig. 4a.

Extracellular marker fluxes

The increment in transepithelial conductance following PLL exposure was further characterised using extracellular tracer flux measurements. [^{14}C]Thiourea and [^{14}C]/[^{3}H]inulin were used as extracellular tracers since such compounds are limited to the paracellular route excluding a small transcytotic component [13].

In strain I MDCK epithelial monolayers under control conditions there was only a limited flux of both [^{14}C]thiourea (Fig. 4a) and [^{3}H]inulin (Fig. 4b) from the apical bathing solution to the basolateral bathing solution (the thiourea flux was approx. 15 times greater than the inulin flux). After apical exposure to PLL there was a dose dependent enhancement of both thiourea (Fig. 4a) and inulin (Fig. 4b) fluxes. A similar dose-dependent increase in apical to basal flux of [^{14}C]inulin was observed in MDCK strain II cells following apical exposure to PLL ([^{14}C]inulin fluxes after 90 min: control 0.92 ± 0.02 (6) pmol cm^{-2} min^{-1}, PLL (10 μg/ml) 6.49 ± 0.61 (6) pmol cm^{-2} min^{-1} ($P <$ 0.0001 vs. control), PLL (50 μg/ml) 21.75 ± 2.55 (6) pmol cm^{-2} min^{-1} ($P < 0.0001$ vs. control)).

Correlation of G_T and transepithelial thiourea flux

Continual G_T measurements were made during the above flux experiments; Fig. 5 shows the correlation between G_T and the apical to basolateral thiourea flux in strain I MDCK monolayers. Two components of transepithelial thiourea transport were evident. A component which appears to be independent of epithelial conductance (i.e., G_T below 0.77 mS cm^{-2}) whilst thiourea flux at conductances above this appear to be directly related to increasing tissue conductance. Similar data were evident for inulin fluxes.

Morphological studies

Effect of PLL on ZO-1 distribution. ZO-1 immunoreactivity in confluent strain I MDCK monolayers was

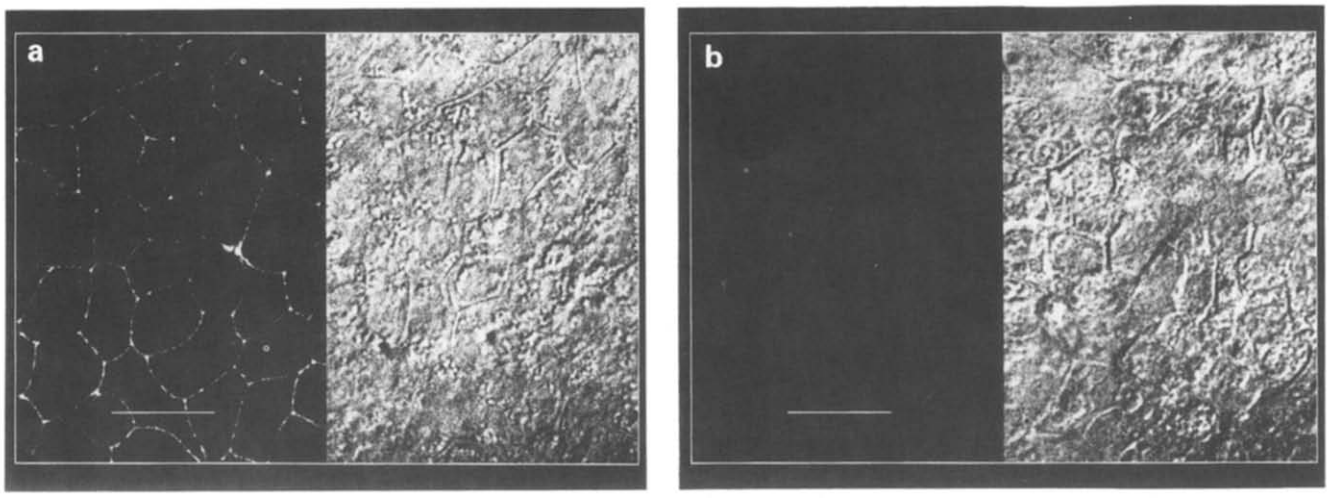

Fig. 6. Effect of PLL on immunofluorescent localisation of ZO-1 in strain I MDCK cell monolayers. Monolayers were exposed to (a) normal Krebs buffer or (b) buffer containing PLL (50 μg/ml) for 15 min before immunofluorescent labelling. The corresponding DIC images are shown for comparison. Bar = 25 μm.

localised to the plasma membrane at areas of cell-to-cell contact (Fig. 6a). Strain II MDCK monolayers displayed a similar staining pattern. Following exposure to PLL (Fig. 6b), junctional ZO-1 immunofluorescence was abolished despite cell morphology being maintained. This suggests that ZO-1 has been lost from its perijunctional sites to become dispersed within the cytoplasm. Similar images were obtained in strain II MDCK epithelia.

Effect of PLL on cytoskeletal actin. When strain I MDCK monolayers were treated with TRITC-phalloidin to stain F-actin, a typical *mosaic* staining pattern was observed (Fig. 7a). Using the confocal microscope to generate XZ (transverse) sections through the monolayer, it was evident that cellular F-actin was concentrated over the entire plasma membrane but was most evident in the lateral cell membranes and in the region of the tight junctional complex. The apical surface of the control epithelium was continuous with no visible breaks. Following exposure to PLL (Fig. 7b), some retraction of cells occurs causing lateral space widening (the double lines between cells (Fig. 7b)). On examination of the XZ sections through these treated monolayers it was observed that lateral space dilatation was also accompanied by discrete breaks in the *zonulae occludentes* at the apical surface. The apical surface membrane of PLL-treated cells appeared to be intact.

PLL exposure is thus accompanied by disruption of *zonulae occludentes* with the appearance of a patent paracellular route.

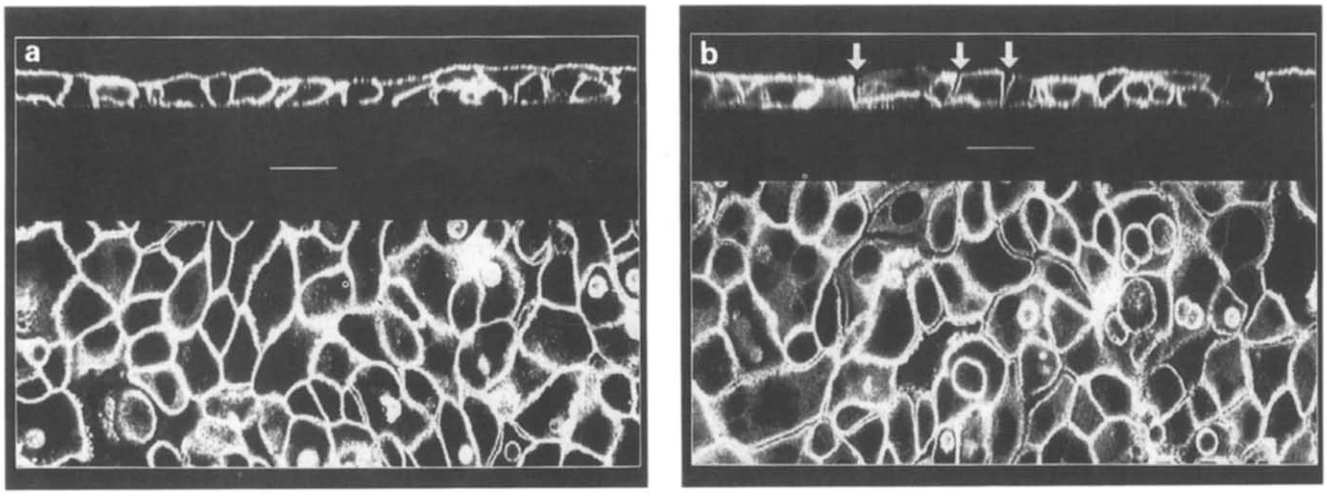

Fig. 7. Effect of PLL on F-actin localisation in strain I MDCK cell monolayers. Monolayers were exposed to (a) normal Krebs buffer or (b) buffer containing PLL (50 μg/ml) for 15 min before incubating with TRITC-phalloidin. XZ confocal images are presented along with conventional images. Following exposure to PLL discrete breaks in the *zonulae occludentes* became evident (arrows). Bar = 25 μm.

Discussion

In cultured monolayers of strain I MDCK cells apical exposure to PLL resulted in a dose-dependent increase in transepithelial conductance which correlated well with increased transepithelial flux of extracellular marker molecules indicating that PLL was acting to enhance paracellular permeability across this epithelium. These effects of PLL are similar to those protamine. These data confirm those of Peterson and Gruenhaupt [4].

However, in *Necturus* gallbladder epithelium, protamine challenge results in a decrease in G_T [1,2,5] which has been attributed to a decrease in apical membrane K^+ conductance [5] preceding intracellular events resulting in reduced permeability of the paracellular pathway. A similar reduction in paracellular permeability has been reported following protamine exposure in renal thin descending limb (TDL) epithelium [3].

Since both *Necturus* gallbladder and TDL epithelia are 'leaky' in nature and MDCK strain I cells form a 'tight' epithelium, we investigated the possibility that the enhancement of paracellular permeability observed in the present study is dependent on the nature of the tight junctional complex. To this end we studied PLL action on strain II MDCK epithelia which have 'leaky', cation selective tight junctions [6,9] similar to those in gallbladder and TDL epithelium. As with the strain I cells, apical PLL challenge in strain II MDCK cells resulted in a rise in G_T with increasing transepithelial flux of extracellular markers. In addition, the selectivity of the paracellular pathway for Na^+ over Cl^- in strain II MDCK cells virtually disappeared after challenge from PLL. Since apical exposure to protamine induced similar effects to PLL in both strain I and II MDCK epithelia it would appear that the observed actions are not specific to PLL. Polycation action on *Necturus* gallbladder and renal TDL are dissimilar to those reported here for strain II MDCK cells. Since these are all 'leaky' epithelia with cation-selective tight junctions it appears that it is not the nature of the junctional complex *per se* which determines polycation action on transepithelial conductance. The protamine-induced decrease in conductance across gallbladder and TDL may be due to a specific property of these epithelia.

The PLL-induced increases in G_T and extracellular marker fluxes in both strain I and II MDCK epithelia were accompanied by alterations in the immunofluorescence distribution of the tight junctional protein, ZO-1. This 225 kDa peripheral membrane protein is located at the cytoplasmic surface of the tight junction [10] and is an important component of the junction. In both strains of MDCK cells, following PLL exposure ZO-1 immunofluorescence disappeared from the junctional area, suggesting that the protein had become dispersed in the cytoplasm. This disipatory action of PLL on ZO-1 was more extreme than that observed following calcium chelation with EGTA in MDCK monolayers [14,15]. Under low calcium conditions it was observed that even when cells had rounded up and become detached ZO-1 remained associated with the plasma membrane [15]. Since ZO-1 has been shown to be concentrated at the points of junctional membrane contact, its complete disappearance suggests that the integrity of the tight junction is compromised in the presence of PLL. This interpretation is strengthened by the observed changes in the pattern of phalloidin binding to cytoskeletal actin. The confocal images of MDCK monolayers exposed to PLL clearly demonstrate breaks in the continuity of the epithelial surface only at points of cell-to-cell contact. This suggests that the apical membrane remains intact and that increased extracellular marker flux is via the opened tight junctional route. Thus it would appear that PLL acts to enhance paracellular permeability through alteration of cytoskeletal structure. It is well known that manipulation of cytoskeletal architecture may modulate paracellular permeability [13]. At present it is unclear whether this cytoskeletal disruption causes the observed changes in ZO-1 distribution or if PLL acts directly on the junctional complex from an extracellular site.

When the time-course for the change in G_T following PLL exposure was studied in greater detail it became apparent that a biphasic phenomenon was occurring. The initial rise in G_T appeared to be associated with the rising phase of the inward I_{sc} change, suggesting a cellular conductance increment. Since the conductance of the apical membrane is considerably smaller than that of the basolateral membrane in control cells [16] it seems likely that the increased cellular conductance is due primarily to an increased conductance in the apical membrane. Such an increase in membrane conductance could account for at least part of the observed PLL-induced rise in G_T. This interpretation of events is strengthened by inspection of the G_T/I_{sc} trajectory plot from the PLL-challenge experiments. This treatment of the data allows a separation of cellular and paracellular conductance elements [11]. The initial inward I_{sc} measured following PLL exposure was associated mainly with an increased cellular conductance; an increased paracellular conductance only becoming apparent subsequently. These data, therefore, suggest that the observed increase in paracellular transport may occur as a result of polycation-mediated cellular action rather than as a direct consequence of polycation action on the junctional complex *per se*.

It is clear from the results of the present study that the reported effects of PLL are general for a number

of polycationic species. However, the number of cationic residues presented to the epithelial surface was found to be important. Random copolymers consisting of varying lysine and alanine residues were most efficient at increasing G_T when the ratio of lysine : alanine was highest. With the exception of poly(L-histidine), all of the cationic polyamino acids investigated caused very similar increases in G_T across the strain I MDCK epithelia. The relatively small change in G_T observed with poly(L-histidine) may have been due to the histidine residues carrying less charge than the lysine, arginine and ornithine residues under the prevailing experimental conditions. Protamine was also less effective in increasing G_T than the polyamino acids but this may be because the protamine molecule is relatively small ($\approx$ 5000 MW) therefore limiting its action. The finding that PLL and poly(D-lysine) had essentially identical effects on MDCK G_T implies that isomerism is not important in this phenomenon.

Similar effects of PLL to those observed in the two MDCK strains were also observed in epithelial cell monolayers of the human colonic adenocarcinoma cell lines T_{84} and HCT-8. It is known that both renal and intestinal brush border membranes possess net negative charge [17] and, thus, it is possible that electrostatic binding is of importance in the induction of the changes in G_T and I_{SC}. This is consistent with the findings of a recent investigation which suggest that polycationic components of human semen (spermine, spermidine) enhance paracellular permeability across colonic mucosa [18]. It has been proposed that this may provide a route for the transmission of viral particles (e.g., HIV virus) from the colorectal lumen into the systemic circulation [18]. However, this action of polyamines on paracellular permeability has been attributed to an induction of collagenase activity [18]. The lack of effect upon basolateral administration of high levels of PLL in the present study is not consistent with this interpretation. Microbial surface interaction (*Salmonella*) has been reported to induce a rapid rise in G_T across MDCK monolayers [19] by an unknown mechanism. Another enteric bacterium (*Vibrio cholerae*) has recently been shown to produce a protein enterotoxin which also causes enhanced paracellular permeation across the intestinal epithelium [20].

The observation that adrenaline stimulation of a Cl^- secretory inward current in MDCK cells was possible following PLL challenge implies that the actions of PLL are not simply toxicological in nature. This was despite G_T having fallen to a fraction of control values. The completeness of the adrenaline response indicates that both the Ca^{2+}-activated K^+ conductance in the basolateral membrane [12] and the cAMP-activated Cl^- conductance in the apical membrane [21] remain intact. It is noteworthy that PLL action resulting in patent tight junctions is restricted to a fraction of the junctions present in the layer.

The mechanism through which PLL interacts with epithelial cells to bring about the observed effects is not known at the present time. It has been shown previously that polycations, including PLL, bind to the cell membranes of erythrocytes [22], Ehrlich ascites tumour cells [23] and toad urinary bladder epithelial cells [24]. Binding of highly charged molecules to integral membrane conductance elements may modify their properties. The predominant conductance present in the apical membrane is, however, an anion selective channel activated by protein kinase A [12,25] which is still functional after PLL exposure. Alternatively, polycation binding may non-specifically alter membrane structure [21,26]. Again, adrenaline stimulation of rheogenic Cl^- secretion argues that gross perturbation of the apical membrane is unlikely. There was no evidence for cellular uptake of polycations in any of the above studies. However, PLL conjugates with albumin and horseradish peroxidase (HRP) have been demonstrated to undergo internalisation into monolayers of L929 mouse fibroblasts [27]. Unconjugated PLL did not enhance uptake of either albumin or HRP implying that the PLL molecule itself was being transported into cells. There is also published evidence that polycations are capable of enhancing cellular uptake of interferon [28] and viral nucleic acids [29]. Thus it is possible that, as well as being adsorbed onto the cell surface, PLL is internalised into cells (possibly via an endocytotic mechanism).

In summary, the results of the present study demonstrate that apical exposure to cationic macromolecules elicits substantial changes in paracellular permeability and electrolyte transport in a variety of cultured epithelia. These polycation-induced alterations to epithelial function are similar between the different epithelial types and appear to be independent of the nature of the tight junctional complex. Enhanced paracellular permeability is accompanied, in MDCK cells, by cytoskeletal alterations and by changes in ZO-1 protein distibution.

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