

# Volume-activated $\text{Cl}^-$ secretion and transepithelial vinblastine secretion mediated by P-glycoprotein are not correlated in cultured human $\text{T}_{84}$ intestinal epithelial layers

G.T.A. McEwan, J. Hunter, B.H. Hirst and N.L. Simmons

*Gastrointestinal Drug Delivery Research Centre and Department of Physiological Sciences, University of Newcastle upon Tyne, Medical School, Newcastle upon Tyne, NE2 4HH, UK*

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The relationship between the P-glycoprotein-mediated vinblastine secretion and cell-swelling activated  $\text{Cl}^-$  secretion (conductance) in intact epithelial layers of human colonic adenocarcinoma  $\text{T}_{84}$  cells has been investigated. Whereas vinblastine secretion is effectively inhibited by  $100\text{ }\mu\text{M}$  1,9-dideoxy-forskolin, volume-stimulated  $\text{Cl}^-$  secretion is unaffected. In contrast,  $100\text{ }\mu\text{M}$  4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) inhibited the volume-stimulated  $\text{Cl}^-$  secretion, but was without effect upon transepithelial vinblastine secretion. In addition, it was noted that some epithelial layers failed to express a volume-stimulated  $\text{Cl}^-$  secretion but maintained a normal level of secretory vinblastine flux.

P-glycoprotein; *MDR*;  $\text{Cl}^-$  secretion; Volume regulation;  $\text{T}_{84}$  cells

## 1. INTRODUCTION

It has recently been demonstrated that permanent transfection of NIH-3T3 fibroblasts and S1 cells with human multidrug resistance (*MDR1*) cDNA results in expression of P-glycoprotein and confers a volume-regulated ATP-dependent  $\text{Cl}^-$  conductance on these cells [1]. Together with P-glycoprotein, the gene product associated with the defect in cystic fibrosis is a member of the ATP-binding cassette (ABC) superfamily of membrane transport proteins [1]. The cystic fibrosis transmembrane conductance regulator protein (CFTR) is a small conductance  $\text{Cl}^-$  channel (5–10 pS) which mediates cAMP-activated epithelial  $\text{Cl}^-$  secretion [2].

Epithelial monolayers of  $\text{T}_{84}$  human colonic adenocarcinoma cells express both *CFTR* [3] and *MDR1* [4] and this is associated with cAMP-stimulated transepithelial  $\text{Cl}^-$  secretion [2,5] and transepithelial vinblastine secretion [4], respectively. P-glycoprotein is expressed in the apical poles of intestinal enterocytes [6] and the existence of polarised secretion of vinblastine by intact  $\text{T}_{84}$  monolayers is consistent with its localisation within the apical membrane [4].

In  $\text{T}_{84}$  colon cells, cAMP-,  $\text{Ca}^{2+}$ - and volume-activated  $\text{Cl}^-$  conductances have all been demonstrated, and this is correlated with the existence of separate  $\text{Cl}^-$  channels with distinct kinetic characteristics [2,7–9]. In

intact  $\text{T}_{84}$  epithelial layers, basal short circuit current (SCC) is minimal, but secretagogues such as vasoactive intestinal peptide (VIP) stimulate an inward transepithelial SCC. The increased SCC is quantitatively accounted for by an increased electrogenic  $\text{Cl}^-$  secretion [10]. The small conductance (5–10 pS) cAMP-activated  $\text{Cl}^-$  channel which is insensitive to inhibition by the stilbene 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) accounts for the substantive portion of the cAMP-induced  $\text{Cl}^-$  current in  $\text{T}_{84}$  cells [2,7]; the cAMP-stimulated SCC in intact  $\text{T}_{84}$  epithelia ( $\text{Cl}^-$  secretion) is also insensitive to DIDS inhibition [5]. The most-studied  $\text{Cl}^-$  channel in epithelial cells is the outward rectifier (30–50 pS) [7,11]. Activity of this channel may be induced in quiescent patches by strong depolarisation and is blocked by extracellular DIDS [2,7,11]. Swelling-induced  $\text{Cl}^-$  currents are kinetically similar to the outwardly rectifying  $\text{Cl}^-$  channel, but it is still unclear whether the two types of current are from a single channel displaying different states or whether separate channels exist [11]. In *MDR1*-transfected cells the volume-activated  $\text{Cl}^-$  conductance was blocked both by DIDS and by the forskolin analogue, 1,9-dideoxy-forskolin (DiD-forskolin), via a cAMP-independent pathway [1]. It has been demonstrated that DiD-forskolin may bind to P-glycoprotein [12] and this analogue has also been shown to inhibit transepithelial vinblastine secretion mediated by P-glycoprotein [13].

It is unknown if swelling-induced  $\text{Cl}^-$  channels contribute to transepithelial (transapical membrane)  $\text{Cl}^-$  current flow in intact  $\text{T}_{84}$  epithelia [7]. The purpose of the present investigation has been to confirm the exis-

Correspondence address: G.T.A. McEwan, Gastrointestinal Drug Delivery Research Centre and Department of Physiological Sciences, University of Newcastle upon Tyne, Medical School, Newcastle upon Tyne, NE2 4HH, UK. Fax: (44) (91) 222 6706.

tence of a volume-activated  $\text{Cl}^-$  conductance in the apical membrane of  $\text{T}_{84}$  epithelium, to determine its pharmacological sensitivity (with particular reference to DIDS and DiD-forskolin), and to compare these data with inhibition of transepithelial vinblastine secretion mediated by P-glycoprotein. Surprisingly, we find that a volume-activated  $\text{Cl}^-$  conductance in  $\text{T}_{84}$  cells does not display the pharmacological sensitivity expected of the  $\text{MDR1-Cl}^-$  channel reported in transfected cells.

## 2. MATERIALS AND METHODS

### 2.1. Cell culture

$\text{T}_{84}$  cells were maintained in serial culture in a 1:1 mixture of Dulbecco's modified Eagles Medium and Hams's F12 with 5% new-born calf serum, 200  $\text{IU}\cdot\text{ml}^{-1}$  of penicillin, 200  $\mu\text{g}\cdot\text{ml}^{-1}$  streptomycin.  $\text{T}_{84}$  cells were grown as epithelial layers upon Anocell 25 mm culture inserts coated with rat-tail collagen using high-density seeding, as previously described [4,14]. Cultures were grown for typically 2 weeks, in 6-well plates at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  with media replacement every 3 days.

### 2.2. Measurement of volume-activated short circuit current (SCC)

Cultured epithelial layers were mounted in Ussing-type chambers maintained at  $37^\circ\text{C}$ , connected to an automatic voltage-clamp via KCl/agar salt-bridges and reversible electrodes (Ag/AgCl for current passage, calomel for voltage sensing) and measurements of open-circuit electrical p.d., transepithelial resistance and short-circuit current (SCC) made in modified Krebs solutions [14]. Isotonic modified Krebs solutions (all mmol/l)  $\text{NaCl}$  70,  $\text{KCl}$  5.4, mannitol 140,  $\text{CaCl}_2$  2.8,  $\text{MgSO}_4$  1.2,  $\text{NaH}_2\text{PO}_4$  0.3,  $\text{KH}_2\text{PO}_4$  0.3, Tris base 14,  $\text{HCl}$  12, glucose 5 (pH 7.4 at  $37^\circ\text{C}$ ) with 1% v/v donor horse serum were switched to hypotonic solutions (composition as above, except mannitol was omitted). In some experiments  $\text{Cl}^-$ -free solutions were used;  $\text{NaCl}$  and  $\text{KCl}$  were replaced by methanesulphonate neutralised by appropriate quantities of  $\text{NaOH}$  and  $\text{KOH}$ , final pH being adjusted with Tris base.  $\text{Ca}(\text{NO}_3)_2$  replaced the  $\text{Cl}^-$  salt.

### 2.3. Measurement of bidirectional transepithelial [ $^3\text{H}$ ]vinblastine sulphate permeabilities

Measurements of transepithelial solute flux were made essentially as described by Hunter *et al.* [4]. Functional epithelial layers in filter cups were washed with 2x3 ml serum-free medium and placed into fresh 6-well plates containing 3 ml serum-free medium (basal solution), a further 3 ml serum-free medium was then pipetted into the upper chamber (apical solution) of the filter cup. Transepithelial resistance was measured following 10 min incubation of the cells at  $37^\circ\text{C}$ , as described above.

The medium on either the apical, or basal side of the monolayers was then removed and replaced with 3 ml serum-free medium containing 10 nM [ $^3\text{H}$ ]vinblastine sulphate as tracer and variable amounts of vinblastine sulphate as indicated in the apical (A) or basal (B) solutions, in the presence or absence of inhibitors as indicated, followed by incubation at  $37^\circ\text{C}$ . In order to measure the bidirectional fluxes of vinblastine sulphate ( $J_{\text{A} \rightarrow \text{B}}$ , flux from apical to basal solutions, and  $J_{\text{B} \rightarrow \text{A}}$ , flux from basal to apical solutions), 100  $\mu\text{l}$  samples of medium from each side of the monolayer were taken at regular intervals,  $^3\text{H}$ -activities in these samples were determined by liquid scintillation counting. Each incubation was performed at least in triplicate. On completion of the flux experiments epithelial integrity was determined by measurement of transepithelial resistance. Measurements of vinblastine flux were determined at concentrations (10 nM – 3  $\mu\text{M}$ )  $\leq K_m$  for transport [13]; to allow ease of comparison, data are expressed as permeabilities, e.g.  $P_{\text{A} \rightarrow \text{B}} = J_{\text{A} \rightarrow \text{B}}/C_A$ , where  $C$  is the vinblastine concentration.

### 2.4. Statistical methods

Data are expressed as mean  $\pm$  S.E.M. (of  $n$  replicates). Tests of

significance of differences between mean values were made using a two-tailed Student's  $t$ -test, or (if stated) Mann-Whitney U test where appropriate.

### 2.5. Materials

[ $^3\text{H}$ ]Vinblastine sulphate was obtained from Amersham International (Little Chalfont, Bucks, UK). All tissue culture media and reagents (Gibco BRL) and tissue culture plastics and Anocell tissue culture inserts (Nunc) were supplied by Life Technologies Ltd. (Paisley, Scotland). 4,4'-Diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) was from Sigma. Stock solutions were made in 1 mM Tris. 1,9-dideoxy-forskolin (DiD-forskolin) was obtained from Calbiochem (Novabiochem, Nottingham, UK). All other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset) or BDH Chemicals Ltd. (Poole, Dorset).

## 3. RESULTS

When intact  $\text{T}_{84}$  epithelial layers were exposed to hypo-osmotic media from the basolateral solution an increased SCC was observed in approximately 62% of  $\text{T}_{84}$  layers (Fig. 1A and B). In responsive layers, the inward SCC increased rapidly to reach peak values of  $10.2 \pm 2.6$  ( $n=4$ )  $\mu\text{A}\cdot\text{cm}^{-2}$ , declining to pre-stimulation values within 5 min (Fig 1A). The peak magnitude of the swelling-induced inward SCC ( $9.8 \pm 0.9$  ( $n=15$ )  $\mu\text{A}\cdot\text{cm}^{-2}$ ) was compared to that observed with 10 nM VIP in the same cell layers ( $4.7 \pm 0.8$  ( $n=15$ )  $\mu\text{A}\cdot\text{cm}^{-2}$ ; Fig. 1B). As noted, in some cases ( $\approx 38\%$ ), hypo-osmotic exposure of  $\text{T}_{84}$  layers did not elicit a SCC response, even with  $\text{T}_{84}$  epithelia from the same culture batch with no discernable difference in initial electrical parameters (Fig. 1B). In these  $\text{T}_{84}$  layers which were unresponsive to hypo-osmotic stimulation, VIP-stimulation of inward SCC was enhanced ( $9.9 \pm 1.6$   $\mu\text{A}\cdot\text{cm}^{-2}$ ;  $P < 0.005$  vs. swelling-responsive tissues, Mann-Whitney U-test).

To test the effects of pharmacological agents on the cell-swelling activated SCC it was necessary to confirm that repeated hypo-osmotic exposures gave rise to an undiminished SCC response. In four responsive layers, the initial response to hypo-osmotic media was  $7.7 \pm 2.3$   $\mu\text{A}\cdot\text{cm}^{-2}$ , whilst a second exposure after 10 min in iso-osmotic media gave  $6.8 \pm 0.7$   $\mu\text{A}\cdot\text{cm}^{-2}$ ; the difference was not statistically significant ( $P < 0.6$  paired data, Mann-Whitney U-test). Media  $\text{Cl}^-$  replacement with methanesulphonate salts, or addition of 100  $\mu\text{M}$  bumetanide, abolish the swelling-activated increment in inward SCC, consistent with this SCC resulting from electrogenic  $\text{Cl}^-$  secretion (data not shown). The swelling-activated SCC was also abolished by DIDS (100  $\mu\text{M}$ ) (Fig. 1A). Comparison of the tissue conductance at the peak of the increment in SCC observed after hypotonic stimulation with that observed in paired DIDS-treated tissue gave a difference (DIDS-sensitive) of  $0.73 \pm 0.25$   $\text{mS}\cdot\text{cm}^{-2}$  ( $n=5$ ). This action of DIDS contrasts to the lack of inhibition with DIDS on the VIP-stimulated  $\text{Cl}^-$  secretion mediated via the small conductance  $\text{Cl}^-$  channel [2,5]. In contrast to the action of DIDS, 100  $\mu\text{M}$  DiD-forskolin had no inhibitory effect upon the swelling-stimulated

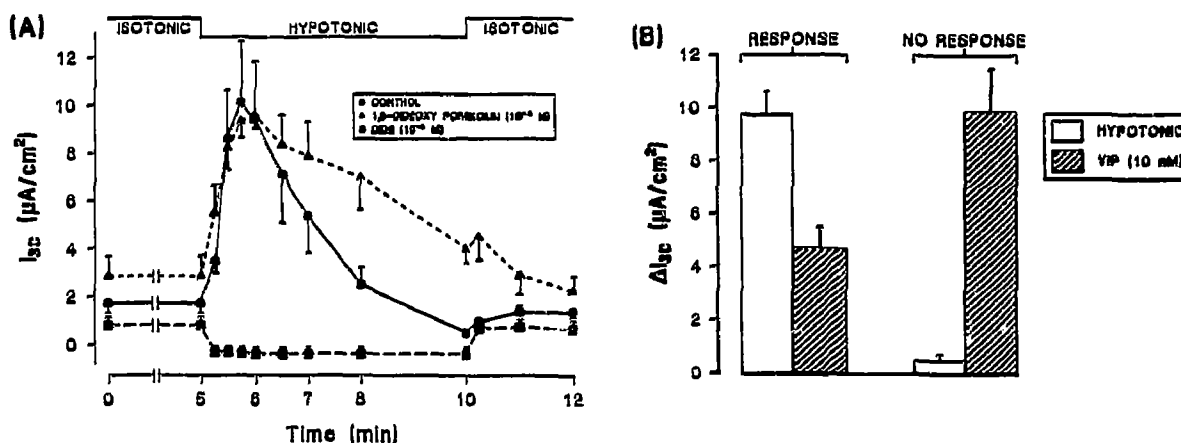


Fig. 1. (A) Effect of hypo-osmolar media applied to the basolateral solutions upon short-circuit current ( $I_{sc}$ ) of intact epithelial monolayers of T<sub>84</sub> cells mounted in Ussing chambers. Hypo-osmolar challenge alone (●;  $n=4$ ), and in the presence of 100  $\mu$ M DIDS applied to both bathing solutions (■;  $n=6$ ) or 100  $\mu$ M dideoxy-forskolin applied to both bathing solutions (▲;  $n=4$ ). Note that there is some potentiation of the SCC response by DiD-forskolin. (B) Comparison of the maximal response of short-circuit current ( $I_{sc}$ ) to 10 nM vasoactive intestinal peptide (VIP) in tissues responsive ( $n=15$ ) and non-responsive ( $n=9$ ) to hypo-osmotic exposure. Results are illustrated as mean with error bars of 1 S.E.M.

Cl<sup>-</sup> secretion. This lack of inhibition contrasts to that observed with DiD-forskolin on volume-activated Cl<sup>-</sup> conductance in *MDR1*-transfected cells [1].

A large asymmetry for unidirectional vinblastine permeability exists across T<sub>84</sub> layers (Fig. 2); this represents net vinblastine secretion from basal to apical cell surfaces. Verapamil (100  $\mu$ M) inhibits net vinblastine secretion by a reduction in  $P_{B \rightarrow A}$  without any significant effect upon  $P_{A \rightarrow B}$ . DiD-forskolin (100  $\mu$ M) is as effective as verapamil, at the same concentration, in inhibiting net vinblastine secretion by reducing  $P_{B \rightarrow A}$ , confirming data previously obtained for vinblastine secretion in MDCK epithelia [13]. In contrast to DiD-forskolin, DIDS has no effect upon vinblastine secretion. The pharmacological profiles for DIDS and DiD-forskolin inhibition of volume-activated Cl<sup>-</sup> secretion and vinblastine secretion in intact native epithelia, are thus distinct.

The observation of responsive and unresponsive tissue phenotypes with respect to volume-activated Cl<sup>-</sup> secretion has allowed us to investigate a possible relationship between these modalities in an alternative way. After scoring epithelial layers for their responsiveness to the volume stimulus, we then proceeded to measure the vinblastine  $P_{B \rightarrow A}$  in the two groups. T<sub>84</sub> epithelial layers lacking volume-activated Cl<sup>-</sup> secretion maintained as large a unidirectional vinblastine permeability ( $P_{B \rightarrow A}$ ) as was observed in those tissues that responded to the volume stimulus (Fig. 2B).

#### 4. DISCUSSION

The present data are consistent with the presence in T<sub>84</sub> epithelia of a volume-activated Cl<sup>-</sup> channel which is able to participate in transepithelial Cl<sup>-</sup> secretion, i.e. is present in the apical membrane. The present data are

thus similar to that reported in other epithelia. In *Necturus* enterocytes, Giraldez *et al.* [15] have shown that a volume-activated Cl<sup>-</sup> conductance is present in the apical membrane, whilst cell swelling has also been reported to stimulate transepithelial Cl<sup>-</sup> secretion in airway epithelium [16].

The Cl<sup>-</sup> secretion stimulated by cell-swelling, reported here, is blocked by extracellular DIDS, in contrast to the VIP-stimulated Cl<sup>-</sup> secretion (mediated by CFTR), which is unaffected by this agent [2,5]. The volume-stimulated Cl<sup>-</sup> secretion is unaffected by 100  $\mu$ M DiD-forskolin, in contrast to the data obtained for the volume-activated Cl<sup>-</sup> conductance in *MDR1*-transfected cell lines [1]. On this basis, the volume-activated Cl<sup>-</sup> secretion reported here is mediated by Cl<sup>-</sup> channel(s) distinct from those in *MDR1*-transfected cells [1]. We confirm, however, that vinblastine secretion, inhibited by verapamil is present in the T<sub>84</sub> epithelia used in this study. Thus it is likely that there is functional expression of P-glycoprotein at the same membrane face at which the volume activated conductance is seen.

The Cl<sup>-</sup>-transport inhibitor DIDS is without effect on vinblastine secretion. There is thus no direct evidence from these studies that DIDS may interact with P-glycoprotein. The existence of a group of tissues in which there is no activation of Cl<sup>-</sup> channels in the apical membrane upon cell swelling has allowed us to test whether vinblastine secretion in these tissues is also absent. However, the unidirectional vinblastine permeability ( $P_{B \rightarrow A}$ ) does not differ between cell-swelling responsive and unresponsive-tissues, indicating that there is no direct relationship between these variables in non-transfected cells.

A small DIDS-sensitive component of tissue conductance ( $\approx 0.75$  mS·cm<sup>-2</sup>) is observed in swollen cells. It is possible to make an estimation of the number of Cl<sup>-</sup>

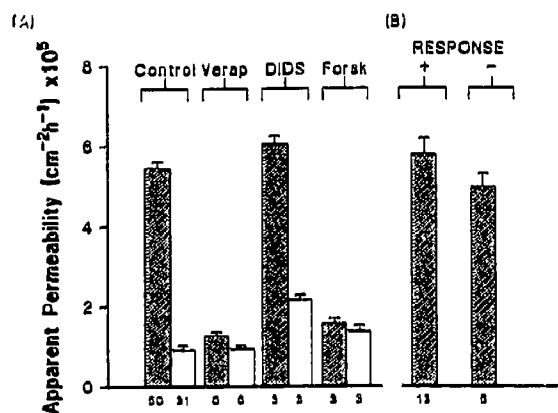


Fig. 2. (A) Bidirectional unidirectional [ $^3\text{H}$ ]vinblastine permeabilities measured across  $T_{84}$  epithelial layers. The apparent permeability of vinblastine in the basal-to-apical ( $P_{B-A} = J_{B-A}/C_B$ ; hatched bars) and in the apical-to-basal ( $P_{A-B} = J_{A-B}/C_A$ ; open bars) was determined under control conditions and in the presence of verapamil (Verap), DIDS and 1,9-dideoxy-forskolin (Forsk), each added at 100  $\mu\text{M}$  to both epithelial surfaces. (B) Measurement of  $P_{A-B}$  for [ $^3\text{H}$ ]vinblastine in layers displaying (+) and failing to display (-) a short-circuit current response to hypo-osmotic exposure (see Fig. 1B). There is no significant difference between these two groups ( $P < 0.34$ ). Results are illustrated as mean with error bars of 1 S.E.M., with number of layers indicated below bars.

channels this may represent from the following relationship;  $G = g \cdot n \cdot P_o$ , where  $G$  is the macroscopic epithelial conductance,  $g$  is the single channel conductance,  $n$  is the volume-stimulated channel number per  $\text{cm}^2$  of epithelium and  $P_o$  is the open-state probability. Assuming a single channel conductance of 50 pS [11], an open state probability of 1.0 in swollen cells and cell area of  $10 \mu\text{m} \times 10 \mu\text{m}$  [ $10^6$  cells per  $\text{cm}^2$ ], this would give a cellular density of just 15  $\text{Cl}^-$  channels per cell. This value is comparable with that observed for whole cell patch clamp data of 75–300 pS/ $100 \mu\text{m}^2$  [11] and implies that the volume-activated  $\text{Cl}^-$  channels are concentrated in the apical membrane [11]. The turnover number of the P-glycoprotein transporter in respect to substrate is unknown, so that similar calculations of the number of P-glycoprotein pump density at the apical membrane cannot yet be made. If the P-glycoprotein does function as a volume-activated  $\text{Cl}^-$  channel, the present data would suggest that the  $\text{Cl}^-$  current carried via P-glycoprotein is small compared to that carried by the

DiD-forskolin-insensitive  $\text{Cl}^-$  conductance reported here. In this case, either the apical membrane density of P-glycoprotein is extremely small ( $\ll 15$  sites/cell), or the kinetic features (e.g. open-state probability or conductance) of the single-channel conductance associated with P-glycoprotein are unusual.

The existence of a reciprocal relationship between the cell-swelling response and the VIP-stimulated inward SCC in identical batches of  $T_{84}$  epithelia, may indicate that the expression of two pharmacologically-distinct  $\text{Cl}^-$  channels at the apical membrane is variable and that unidentified factors are operative in controlling the relative tissue response to cAMP and volume stimuli.

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