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Volume-activated Cl^- secretion and transepithelial vinblastine secretion mediated by P-glycoprotein are not correlated in cultured human 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 Cl^- secretion (conductance) in intact epithelial layers of human colonic adenocarcinoma T_{84} cells has been investigated. Whereas vinblastine secretion is effectively inhibited by 100 μM 1,9-dideoxy-forskolin, volume-stimulated Cl^- secretion is unaffected. In contrast, 100 μM 4,4'-diisothiocyanostilbene-2,2'disulphonic acid (DIDS) inhibited the volume-stimulated 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 Cl^- secretion but maintained a normal level of secretory vinblastine flux.

P-glycoprotein; *MDR*; Cl^- secretion; Volume regulation; T_{84} cells

1. INTRODUCTION

It has recently been demonstrated that permanent transfection of NIH-3T3 fibroblasts and S1 cells with human multidrug resistance (*MDR*1) cDNA results in expression of P-glycoprotein and confers a volume-regulated ATP-dependent 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 Cl^- channel (5–10 pS) which mediates cAMP-activated epithelial Cl^- secretion [2].

Epithelial monolayers of T_{84} human colonic adenocarcinoma cells express both *CFTR* [3] and *MDR*1 [4] and this is associated with cAMP-stimulated transepithelial 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 T_{84} monolayers is consistent with its localisation within the apical membrane [4].

In T_{84} colon cells, cAMP-, Ca^{2+}- and volume-activated Cl^- conductances have all been demonstrated, and this is correlated with the existence of separate Cl^- channels with distinct kinetic characteristics [2,7–9]. In intact 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 Cl^- secretion [10]. The small conductance (5–10 pS) cAMP-activated Cl^- channel which is insensitive to inhibition by the stilbene 4,4'-diisothiocyanantostilbene-2,2'-disulphonic acid (DIDS) accounts for the substantive portion of the cAMP-induced Cl^- current in T_{84} cells [2,7]; the cAMP-stimulated SCC in intact T_{84} epithelia (Cl^- secretion) is also insensitive to DIDS inhibition [5]. The most-studied 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 Cl^- currents are kinetically similar to the outwardly rectifying 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 *MDR*1-transfected cells the volume-activated 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 Cl^- channels contribute to transepithelial (transapical membrane) Cl^- current flow in intact 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 Cl^- conductance in the apical membrane of 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 Cl^- conductance in T_{84} cells does not display the pharmacological sensitivity expected of the *MDR*1-Cl^- channel reported in transfected cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

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 IU·ml^{-1} of penicillin, 200 μg·ml^{-1} streptomycin. 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°C, 5% 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°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) NaCl 70, KCl 5.4, mannitol 140, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, Tris base 14, HCl 12, glucose 5 (pH 7.4 at 37°C) with 1% v/v donor horse serum were switched to hypotonic solutions (composition as above, except mannitol was omitted). In some experiments Cl^--free solutions were used; NaCl and KCl were replaced by methanesulphonate neutralised by appropriate quantities of NaOH and KOH, final pH being adjusted with Tris base. $Ca(NO_3)_2$ replaced the Cl^- salt.

2.3. *Measurement of bidirectional transepithelial [^{3}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 2×3 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°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}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°C. In order to measure the bidirectional fluxes of vinblastine sulphate ($J_{A\text{-}B}$, flux from apical to basal solutions, and $J_{B\text{-}A}$, flux from basal to apical solutions), 100 μl samples of medium from each side of the monolayer were taken at regular intervals, ^{3}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 μM) $\leq K_m$ for transport [13]; to allow ease of comparison, data are expressed as permeabilities, e.g. $P_{A\text{-}B} = J_{A\text{-}B}/C_A$, where C is the vinblastine concentration.

2.4. *Statistical methods*

Data are expressed as mean ±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}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 T_{84} epithelial layers were exposed to hypo-osmotic media from the basolateral solution an increased SCC was observed in approximately 62% of T_{84} layers (Fig. 1A and B). In responsive layers, the inward SCC increased rapidly to reach peak values of 10.2±2.6 (n=4) μA·cm^{-2}, declining to pre-stimulation values within 5 min (Fig 1A). The peak magnitude of the swelling-induced inward SCC (9.8±0.9 (n=15) μA·cm^{-2}) was compared to that observed with 10 nM VIP in the same cell layers (4.7±0.8 (n=15) μA cm^{-2}; Fig. 1B). As noted, in some cases (≈ 38%), hypo-osmotic exposure of T_{84} layers did not elicit a SCC response, even with T_{84} epithelia from the same culture batch with no discernable difference in initial electrical parameters (Fig. 1B). In these T_{84} layers which were unresponsive to hypo-osmotic stimulation, VIP-stimulation of inward SCC was enhanced (9.9±1.6 μA·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±2.3 μA·cm^{-2}, whilst a second exposure after 10 min in iso-osmotic media gave 6.8±0.7 μA·cm^{-2}; the difference was not statistically significant ($P < 0.6$ paired data, Mann-Whitney U-test). Media Cl^- replacement with methanesulphonate salts, or addition of 100 μM bumetanide, abolish the swelling-activated increment in inward SCC, consistent with this SCC resulting from electrogenic Cl^- secretion (data not shown). The swelling-activated SCC was also abolished by DIDS (100 μM) (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 ± 0.25 mS·$^{-2}$ (n=5). This action of DIDS contrasts to the lack of inhibition with DIDS on the VIP-stimulated Cl^- secretion mediated via the small conductance Cl^- channel [2,5]. In contrast to the action of DIDS, 100 μM DiD-forskolin had no inhibitory effect upon the swelling-stimulated

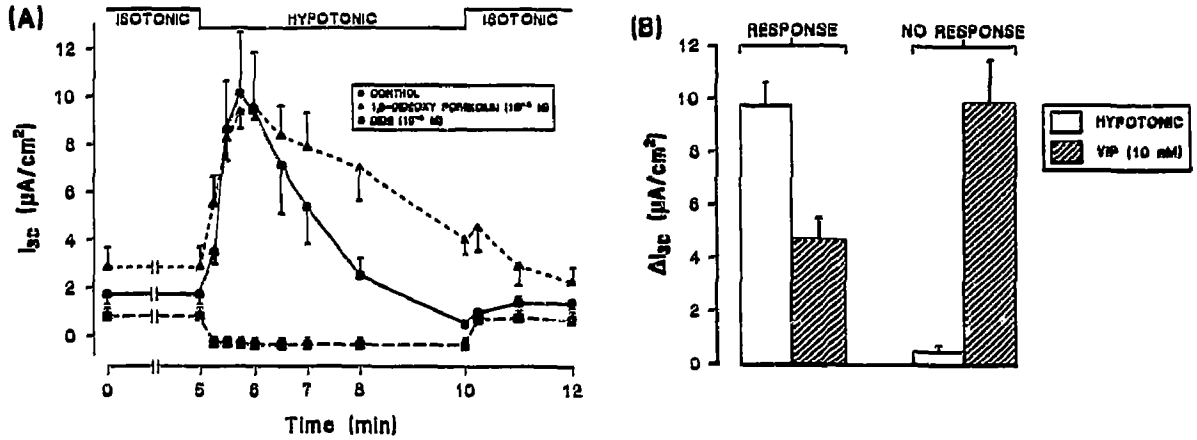

Fig. 1. (A) Effect of hypo-osmolar media applied to the basolateral solutions upon short-circuit current (I_{sc}) of intact epithelial monolayers of T_{84} cells mounted in Ussing chambers. Hypo-osmolar challenge alone (●; n=4), and in the presence of 100 μM DIDS applied to both bathing solutions (■; n=6) or 100 μM 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^- secretion. This lack of inhibition contrasts to that observed with DiD-forskolin on volume-activated Cl^- conductance in *MDR*1-transfected cells [1].

A large assymmetry for unidirectional vinblastine permeability exists across T_{84} layers (Fig. 2); this represents net vinblastine secretion from basal to apical cell surfaces. Verapamil (100 μM) inhibits net vinblastine secretion by a reduction in $P_{B\text{-}A}$ without any significant effect upon $P_{A\text{-}B}$. DiD-forskolin (100 μM) is as effective as verapamil, at the same concentration, in inhibiting net vinblastine secretion by reducing $P_{B\text{-}A}$, 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^- 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^- 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 $P_{B\text{-}A}$ in the two groups. T_{84} epithelial layers lacking volume-activated Cl^- secretion maintained as large a unidirectional vinblastine permeability ($P_{B\text{-}A}$) 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_{84} epithelia of a volume-activated Cl^- channel which is able to participate in transepithelial Cl^- 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^- conductance is present in the apical membrane, whilst cell swelling has also been reported to stimulate transepithelial Cl^- secretion in airway epithelium [16].

The Cl^- secretion stimulated by cell-swelling, reported here, is blocked by extracellular DIDS, in contrast to the VIP-stimulated Cl^- secretion (mediated by CFTR), which is unaffected by this agent [2,5]. The volume-stimulated Cl^- secretion is unaffected by 100 μM DiD-forskolin, in contrast to the data obtained for the volume-activated Cl^- conductance in *MDR*1-transfected cell lines [1]. On this basis, the volume-activated Cl^- secretion reported here is mediated by Cl^- channel(s) distinct from those in *MDR*1-transfected cells [1]. We confirm, however, that vinblastine secretion, inhibited by verapamil is present in the T_{84} 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^--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^- 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 ($P_{B\text{-}A}$) 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^{-2}) is observed in swollen cells. It is possible to make an estimation of the number of Cl^-

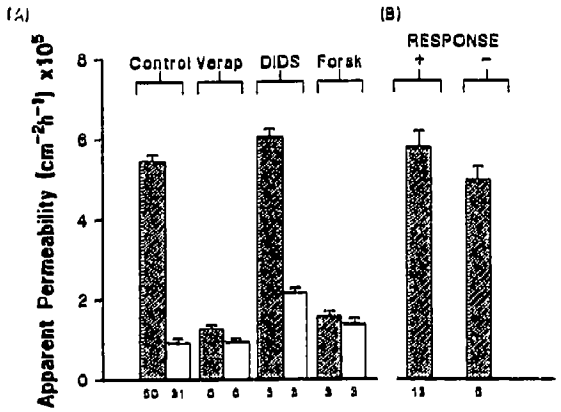

Fig. 2. (A) Bidirectional unidirectional [^{3}H]vinblastine permeabilities measured across T_{84} epithelial layers. The apparent permeability of vinblastine in the basal-to-apical ($P_{B\text{-}A} = J_{B\text{-}A}/C_B$; hatched bars) and in the apical-to-basal ($P_{A\text{-}B} = J_{A\text{-}B}/C_A$; 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 μM to both epithelial surfaces. (B) Measurement of $P_{A\text{-}B}$ for [^{3}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 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 μm × 10 μm [10^{6}cells per cm^2), this would give a cellular density of just 15 Cl^- channels per cell. This value is comparable with that observed for whole cell patch clamp data of 75–300 pS/100 μm^2 [11] and implies that the volume-activated 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 Cl^- channel, the present data would suggest that the Cl^- current carried via P-glycoprotein is small compared to that carried by the DiD-forskolin-insensitive Cl^- conductance reported here. In this case, either the apical membrane density of P-glycoprotein is extremely small (≪ 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 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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