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# POLARIZED EFFLUX OF 2′,7′-BIS(2-CARBOXYETHYL)-5(6)-CARBOXYFLUORESCEIN FROM CULTURED EPITHELIAL CELL MONOLAYERS

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**Abstract**—We have investigated the polarity of the efflux of the intracellular pH fluorochrome 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) from layers of epithelial Madin–Darby canine kidney (MDCK, Strains I and II) and human intestinal (Caco-2, HCT-8 and $T_{84}$) cells grown on porous membranes. In Strain I MDCK cells, BCECF efflux was effectively reduced by indomethacin (50% inhibition with 100 μM) and 5-nitro-2-(3-phenylpropyl-amino)-benzoate (NPPB; 50% inhibition with 10 μM). Replacement of external $Cl^-$ with bromide, iodide or nitrate did not alter BCECF efflux, while substitution with methanesulphonate resulted in a small but significant reduction. All five cell lines form confluent epithelial layers when grown on porous membranes. Efflux of BCECF from Strain I MDCK epithelial layers into the apical solution was approximately three times greater than into the basal solution. Addition of indomethacin to the apical solution attenuated efflux into the apical but not the basal solution, while basal indomethacin was effective against basal efflux. NPPB has a similar specificity of action. Adrenaline, a stimulant of electrogenic $Cl^-$ secretion, did not alter the pattern of BCECF efflux. BCECF efflux was also polarized, with apical efflux greater than basal efflux, in MDCK Strain II and Caco-2 epithelial layers. In contrast, BCECF efflux into the basal and apical media was equivalent in layers formed from HCT-8 and $T_{84}$ cells. However, indomethacin reduced efflux in all five epithelial lines, although the relative sensitivities of the apical and basal efflux rates to indomethacin varied, as did the sensitivity to the sidedness of application of indomethacin. In MDCK and HCT-8 epithelial layers, transepithelial vinblastine secretion mediated by P-glycoprotein was not inhibited by indomethacin. The data are consistent with the hypothesis that BCECF efflux is a manifestation of a novel ATP-dependent xenobiotic secretory efflux mechanism in renal and gastrointestinal epithelia. The factors regulating the polarity of BCECF efflux, both the indomethacin-sensitive and -insensitive components, have yet to be elucidated.

The fluorochrome 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyflurorescein (BCECF†) is widely used as an indicator of intracellular pH, but its use may be limited by dye loss from cells [1]. We have reported recently that BCECF loss from human intestinal adenocarcinoma epithelial cells (HCT-8 and $T_{84}$) and renal Madin–Darby canine kidney (MDCK) cells is not simply passive leakage, but is mediated via an ATP-dependent transport mechanism and is inhibitable with a novel pharmacological profile [2–4].

The multidrug resistance gene (*MDR*1) product, P-glycoprotein, functions as an ATP-dependent drug efflux pump [5]. In addition to typical cytotoxic drugs such as vinblastine, daunomycin and actinomycin D, several fluorochromes, including rhodamine 123 and the $Ca^{2+}$ indicator fluo-3, are substrates for P-glycoprotein [6–8]. Although showing some similarities to P-glycoprotein-mediated drug efflux, BCECF efflux demonstrates a distinct pharmacological profile [2, 4]. The functional expression of P-glycoprotein in several epithelial cells, including renal MDCK and intestinal HCT-8 and $T_{84}$, results in polarized secretion of substrates such as vinblastine from the apical membrane [9–11]. This has led to a model for P-glycoprotein function as a cellular secretory detoxifying mechanism in epithelia [9]. To investigate the hypothesis that BCECF efflux is a manifestation of a similar cellular secretory detoxifying mechanism in the kidney and intestine, we have characterized the polarity of BCECF efflux in a variety of epithelial cells grown as confluent layers on porous membranes.

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† Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; MDCK, Madin–Darby canine kidney; NPPB, 5-nitro-2-(3-phenylpropyl-amino)-benzoate; DIDS, 4,4′-diisothiocyano-2,2′-disulphonicstilbene; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulphonic stilbene.

## MATERIALS AND METHODS

*Cell culture.* The MDCK cells, both Strain I which displays a high transepithelial electrical resistance and the low resistance Strain II [12], were routinely grown in Eagle's minimum essential medium with non-essential amino acids (1% v/v), 4 mM L-glutamine and 10 μg/mL kanamycin supplemented with 2% foetal calf serum and 8% donor horse serum. Caco-2 cells [13] were routinely grown in Dulbecco's modified Eagle's medium with non-

essential amino acids (1% v/v) and 2 mM glutamine supplemented with 10% foetal calf serum. HCT-8 cells [14] were maintained in RPMI 1640 with 10% horse serum, 2 mM glutamine and 1 mM sodium pyruvate. $T_{84}$ cells [15] were maintained in serial culture in a 1:1 mixture of Dulbecco's modified Eagle's medium and Hams's F12 with 5% new-born calf serum, 200 IU/mL of penicillin, 200 $\mu$g/mL streptomycin. All cells were maintained in a humidified atmosphere of 5% $CO_2$ in air at 37°. Single-cell suspensions for serial passage were prepared by incubation in 0.1–0.05% trypsin, 0.02% EDTA in $Ca^{2+}$- and $Mg^{2+}$-free Earle's Balanced Salt Solution at 37° and trituration through a wide-bore (2-mm i.d.) needle.

For studies of BCECF efflux from MDCK cells grown on plastic, cells were seeded into 24-well plates at a density of $1.5 \times 10^5$ cells/well and allowed to form monolayers over the next 2–4 days. For studies of polarity of efflux from confluent epithelial cell layers, cells were seeded at high density into either ($0.5 \times 10^6$ cells) 10-mm diameter Anopore inorganic (MDCK, Caco-2 and $T_{84}$; Nunc, Life Technologies) or ($3 \times 10^6$ cells) 24-mm diameter polycarbonate (HCT-8; Transwell, Costar) membrane tissue culture inserts. Inserts were precoated with rat-tail collagen for HCT-8 and $T_{84}$ cells and then cultured at 37° and 5% $CO_2$, for 3–4 (MDCK), 11–14 (Caco-2) or 7–11 (HCT-8, $T_{84}$) days, before use. The formation of functional epithelial layers was monitored by the development of transepithelial electrical resistance as measured using WPI Evometer fitted with "chopstick" electrodes to allow transepithelial current passage and potential sensing. Cell monolayers were used when the transepithelial resistance exceeded 1000 (MDCK), 200 (Caco-2) or 700 (HCT-8 and $T_{84}$) $\Omega \cdot cm^2$.

*Measurement of BCECF efflux.* Cell monolayers were washed twice with 1 mL PBS before loading with BCECF by the addition of the 10–20 $\mu$M of the non-fluorescent cell-permeant acetoxymethyl ester form of BCECF (BCECF-AM) and incubation for 60 min at 37°. After further washing of the cell layers, BCECF efflux was then monitored at 37°, 95% air/5% $CO_2$ in Eagle's minimum essential medium and quantified, after diluting the samples 1:100 in phosphate-buffered saline (pH 9.0), as fluorescence at 530 nm (500 nm in excitation) in a Perkin–Elmer LS-5 spectrofluorimeter. Remaining intracellular BCECF was released by lysis with 0.1% (v/v) Triton X-100, and BCECF efflux was expressed as a percentage of the total initial cellular BCECF. Test drugs were investigated for their influence on BCECF fluorescence and, where necessary, fluorescence was corrected using standard curves of BCECF with and without drug.

*Measurement of bidirectional transepithelial [$^3$H]-vinblastine sulphate fluxes.* Measurements of transepithelial solute flux were made essentially as described by Hunter *et al.* [10, 11]. MDCK or HCT-8 cells were grown as epithelial layers by high-density seeding ($3 \times 10^6$ cells) onto permeable filter matrices [Anopore 25-mm (MDCK) or collagen-coated Transwell 24.5-mm (HCT-8) culture inserts]. Functional epithelial layers in filter cups were washed with $2 \times 3$ mL serum-free medium and placed into fresh six-well plates containing 3 mL serum-free medium (basal solution); a further 3 mL serum-free medium were then transferred into the upper chamber (apical solution) of the filter cup. Transepithelial resistance was measured following 10 min incubation of the cells at 37°, 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 that on the contralateral side replaced with 3 mL serum-free medium containing 10 nM vinblastine sulphate, in the presence or absence of varying concentrations of indomethacin or verapamil, followed by incubation at 37°. In order to measure the bidirectional fluxes of vinblastine sulphate ($J_{A-B}$, flux from apical to basal solutions and $J_{B-A}$, flux from basal to apical solutions), 100-$\mu$L 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.

*Effect of pharmacological agents.* Stock solutions of agents were freshly prepared in serum-free medium or in dimethyl sulphoxide followed by dilution in serum-free medium, with brief sonication to aid dissolution, and the pH was adjusted to 7.4. Control wells were included in each experiment consisting of serum-free medium with or without dimethyl sulphoxide (0.1% v/v) as a vehicle control.

*Materials.* BCECF and BCECF-AM were supplied by Life Technologies (Paisley, U.K.). Pharmacological agents were supplied by the Sigma Chemical Co. (Poole, U.K.). 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB) was a gift from SmithKline Beecham Pharmaceuticals (Welwyn, U.K.). All cell culture media and supplements (Gibco BRL) and plastic cell culture flasks and plates (Nunc) were purchased from Life Technologies Ltd.

*Treatment of results.* Results are expressed as means ± 1 SE (number of wells). Significance of difference was investigated by the Student's *t*-test using non-normalized data, with significance level set at $P \leq 0.05$.

## RESULTS

### *Modulation of BCECF efflux in MDCK cells grown on plastic*

We have described previously in detail the pharmacological profile for inhibition of BCECF efflux from human HCT-8 intestinal epithelial cells, including sensitivity to inhibition by blockers of anion channels such as NPPB and indomethacin but relative insensitivity to 4,4′-diisothiocyano-2,2′-disulphonic stilbene (DIDS) and 4-acetamido-4′-isothiocyano-2,2′-disulphonic stilbene (SITS) [4]. We have examined the sensitivity of BCECF efflux to these agents in Strain I MDCK cells. SITS and DIDS had little effect on BCECF efflux at concentrations ≤ 1 mM (Fig. 1). Even with prolonged incubations (5 hr) BCECF efflux in the presence of

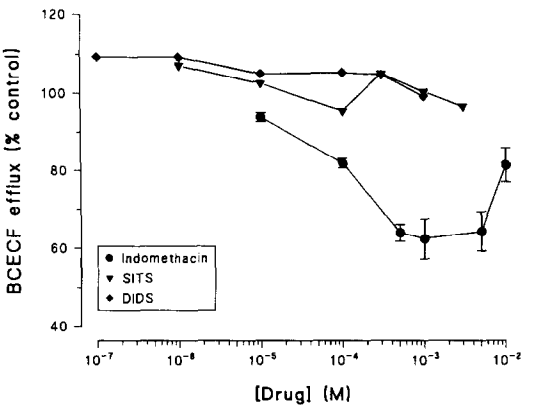


Fig. 1. Concentration–response relationship for inhibition of BCECF efflux over 3 hr by indomethacin (N = 4), SITS (N = 2) and DIDS (N = 2) in Strain I MDCK cells grown on plastic. BCECF efflux is expressed as a percentage of control efflux. Results are illustrated as means ± 1 SE (or range for N = 2; range is within symbol).

1 mM SITS did not significantly reduce BCECF efflux [87 ± 2% (N = 9) of control efflux]. In contrast, indomethacin caused a marked reduction in BCECF efflux (Fig. 1). Maximal inhibition of BCECF efflux (~ 40% inhibition) was observed with concentrations of indomethacin of 0.5–5 mM, with a concentration required for half-maximal inhibition of ~ 100 $\mu$M. The concentration–response relationship for inhibition of BCECF efflux by indomethacin was an inverted bell shape: 10 mM indomethacin resulted in only half-maximal inhibition (Fig. 1). High concentrations of indomethacin are associated with cell lysis [3]. The $Cl^-$ channel blocker NPPB was also an effective inhibitor of BCECF efflux in MDCK cells. BCECF efflux was inhibited by ~50%

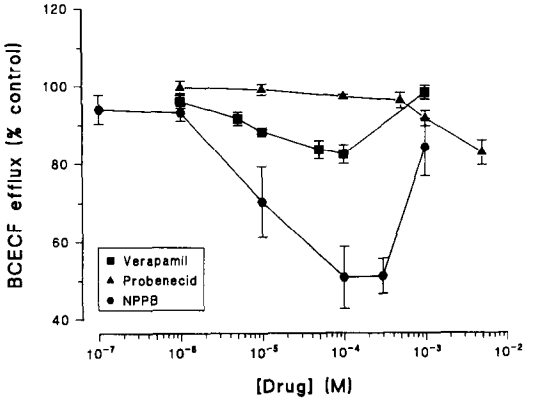


Fig. 2. Concentration–response relationship for inhibition of BCECF efflux over 3 hr by verapamil (N = 4), probenecid (N = 3) and NPPB (N = 5) in Strain I MDCK cells grown on plastic. BCECF efflux is expressed as a percentage of control efflux. Results are illustrated as means ± 1 SE.

with NPPB 0.1–0.5 mM and the concentration required for half-maximal inhibition was ~ 10 $\mu$M (Fig. 2). Thus, BCECF efflux was more sensitive to inhibition by NPPB than indomethacin. As was observed with indomethacin, the concentration–response curve for inhibition of BCECF efflux by NPPB was an inverted bell shape: 1 mM NPPB did not significantly inhibit BCECF efflux (Fig. 2).

Two other agents reported to reduce BCECF efflux in HCT-8 cells were also effective in MDCK cells. Verapamil caused a small but significant reduction in BCECF efflux which was maximal at 50–100 $\mu$M and reversed at 1 mM (Fig. 2), a pattern similar to that observed in HCT-8 cells [4]. Probenecid reduced BCECF efflux only at a concentration of $\geq$ 1 mM (Fig. 2). BCECF efflux was unaffected by reserpine or nifedipine used at 1 mM (BCECF efflux 98 ± 2% and 99 ± 1% (N = 9) of control flux over 5 hr, respectively).

### *Effect of* $Cl^-$ *replacement on BCECF efflux*

BCECF efflux was compared in Strain I MDCK cells with external chloride substituted for a variety of other anions. Over a 3-hr period, 87 ± 1% (N = 8) of the initial BCECF left the cells in a normal Krebs solution ($[Cl^-]$ = 148 mM). Addition of 0.5 mM indomethacin to this solution reduced the BCECF efflux to 46 ± 2% (N = 8). Replacing the chloride in the Krebs solution with iodide, bromide or nitrate did not significantly alter BCECF efflux: 86 ± 1%, 91 ± 0% and 91 ± 1% (N = 8), respectively. Replacement of external chloride by methanesulphonate resulted in a small significant reduction in BCECF efflux to 77 ± 1% (N = 8).

### *Polarity of BCECF efflux in MDCK cells*

To study the polarity of BCECF efflux, Strain I MDCK cells were seeded at high density on to porous tissue culture inserts (Nunc 10-mm Anopore inserts) and allowed to form confluent monolayers. The formation of the epithelial monolayers was judged by visual observation and confirmed by the development of mean transepithelial electrical resistances of 3060 ± 270 (N = 24) $\Omega \cdot cm^2$.

BCECF was released into both the apical and basal solutions bathing MDCK cell layers in a time-dependent manner. Over the same time period (5 hr), free BCECF added to either the basal or apical solutions did not traverse the cell layers to appear in detectable quantities in the contralateral solution. The appearance of BCECF in the apical solution exceeded that in the basal solution at all times investigated (Fig. 3). After 5 hr, 70 ± 2% (N = 11) of the BCECF was in the apical solution while only 23 ± 2% (N = 12) was in the basal solution.

### *Pharmacological modulation of polarized BCECF efflux in MDCK cells*

The nature of the polarized efflux of BCECF was investigated using the pharmacological tools identified earlier. Indomethacin, 0.5 mM, present in the apical solution attenuated BCECF efflux into the apical solution but had little effect on the smaller rate of BCECF efflux into the basal solution (Fig. 3). In contrast, addition of indomethacin to the basal solution had little effect on BCECF efflux into the

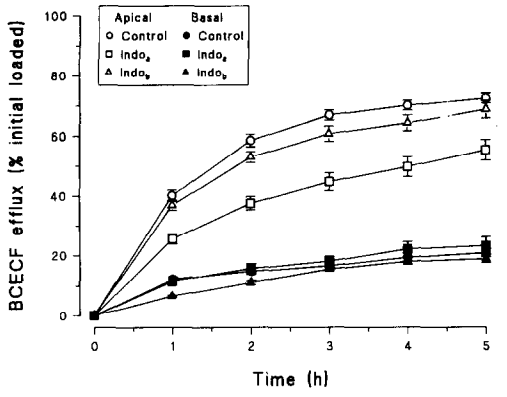


Fig. 3. Polarity of BCECF efflux from Strain I MDCK epithelial layers as a function of time. MDCK cells were loaded with BCECF and then transferred to serum-free medium (N = 7) or serum-free medium containing indomethacin 0.5 mM in either the apical (N = 8) or basal (N = 8) solutions. BCECF efflux is expressed as a percentage of the total initial BCECF in the layers. Results are illustrated as means ± 1 SE.

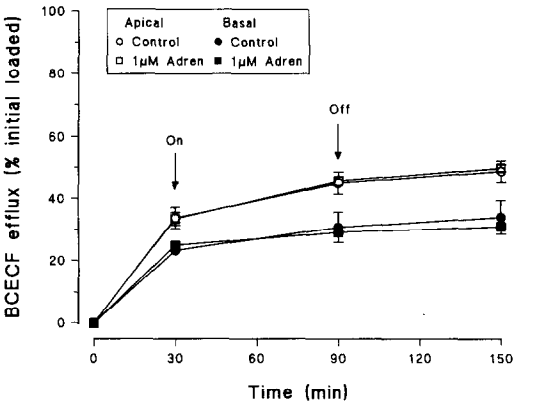


Fig. 5. Polarity of BCECF efflux from Strain I MDCK epithelial layers as a function of time in the presence (N = 4) or absence (N = 2) of adrenaline. Adrenaline 1 μM was present in the basal solution from 30-90 min. BCECF efflux is expressed as a percentage of the total initial BCECF in the layers. Results are illustrated as means ± 1 SE (or range for N = 2).

apical solution but reduced the efflux into the basal solution at early time points (Fig. 3). NPPB, 0.1 mM, had a similar effect on polarized BCECF efflux (Fig. 4). Addition of NPPB to the apical solution reduced efflux of BCECF into the apical solution and into the basal solution at early time points. Addition of NPPB to the basal solution reduced BCECF efflux into the basal solution at early time points but did not significantly affect apical efflux.

Adrenaline is a recognised stimulant of $Cl^-$ secretion, as indicated by an increase in the transepithelial short-circuit current in MDCK cell layers [16]. We investigated whether adrenaline treatment affected BCECF efflux in MDCK cell monolayers. Adrenaline, 1 μM, was added to the basal solution of BCECF-loaded MDCK epithelial layers after 30 min and left in contact with the cells for 60 min before removal. BCECF efflux into both the apical and basal solutions was unaffected during this period (Fig. 5). In another protocol, adrenaline, 0.1 μM, was present in the basal solution throughout the whole 3-hr experimental period. In these latter experiments, after 3 hr, BCECF efflux into the apical solution was similar in the presence [62 ± 2% (N = 5)] or absence [65 ± 1% (N = 5)] of adrenaline, while in parallel experiments, indomethacin, 0.5 mM apical, effectively reduced the apical efflux [42 ± 1% (N = 2)]. Basal efflux of BCECF was similar under control conditions [26 ± 1% (N = 5)] and in the presence of adrenaline [28 ± 2% (N = 5)] or indomethacin [26 ± 0% (N = 2)].

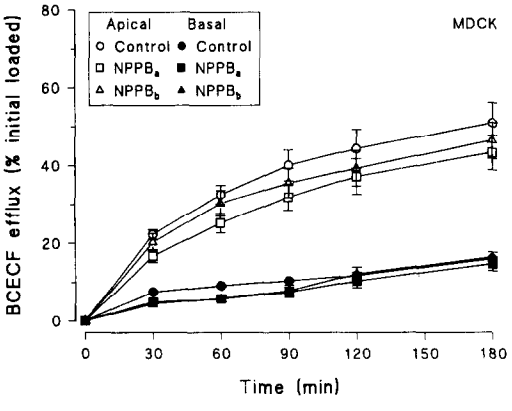


Fig. 4. Polarity of BCECF efflux from Strain I MDCK epithelial layers as a function of time. MDCK cells were loaded with BCECF and then transferred to serum-free medium (N = 8) or serum-free medium containing NPPB 0.1 mM in either the apical (N = 8) or basal (N = 8) solutions. BCECF efflux is expressed as a percentage of the total initial BCECF in the layers. Results are illustrated as means ± 1 SE.

*Polarity of BCECF efflux in other epithelial cell layers*

Indomethacin-sensitive BCECF efflux has been noted in several epithelial cell lines, including human intestinal epithelial cells such as HCT-8 and $T_{84}$, in addition to MDCK [2]. We, therefore, investigated several other epithelial cell lines for evidence of polarity of BCECF efflux.

In the Strain II MDCK cell layers, an example of a "leaky" epithelium, apical efflux of BCECF was again greater than basal efflux, although the difference between the efflux into the two solutions was much less than observed with Strain I MDCK layers (Fig. 6). BCECF efflux in Strain II MDCK cells was sensitive to inhibition with indomethacin, but the pattern of inhibition differed from that observed in Strain I. Apical efflux of BCECF was again sensitive to apical addition of indomethacin, while there was also some sensitivity to basal addition of indomethacin. In contrast to the results in Strain

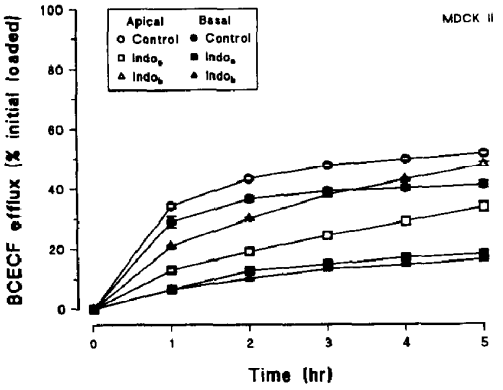


Fig. 6. Polarity of BCECF efflux from Strain II MDCK epithelial layers as a function of time. MDCK cells were loaded with BCECF and then transferred to serum-free medium or serum-free medium containing indomethacin 0.5 mM in either the apical or basal solutions. BCECF efflux is expressed as a percentage of the total initial BCECF in the layers. Results are illustrated as means ± 1 SE (N = 4).

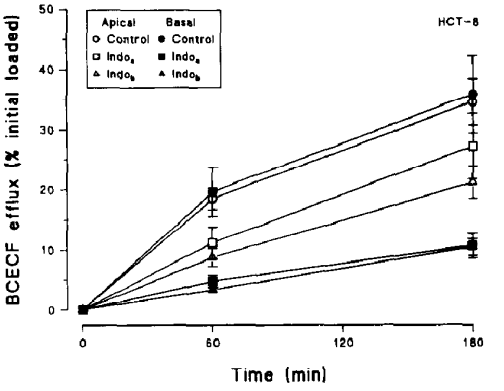


Fig. 8. Polarity of BCECF efflux from HCT-8 epithelial layers as a function of time. HCT-8 cells were loaded with BCECF and then transferred to serum-free medium or serum-free medium containing indomethacin 0.5 mM in either the apical or basal solutions. BCECF efflux is expressed as a percentage of the total initial BCECF in the layers. Results are illustrated as means ± 1 SE (N = 4).

I, basal efflux of BCECF in Strain II MDCK layers was sensitive to indomethacin and this was similar whether the indomethacin was present in the basal or apical solutions (Fig. 6).

In Caco-2 human intestinal cell layers, BCECF efflux was also polarized, being greater into the apical as compared with the basal solution; 57 ± 1 as compared with 19 ± 1% (N = 8) over 3 hr, respectively (Fig. 7). Apical addition of 0.5 mM indomethacin to Caco-2 layers reduced apical BCECF efflux to 43 ± 1% (N = 8) at 3 hr. Basal BCECF efflux was also reduced by 0.5 mM indomethacin, apical or basal, to 9 ± 1% (N = 8 or 7, respectively) (Fig. 7).

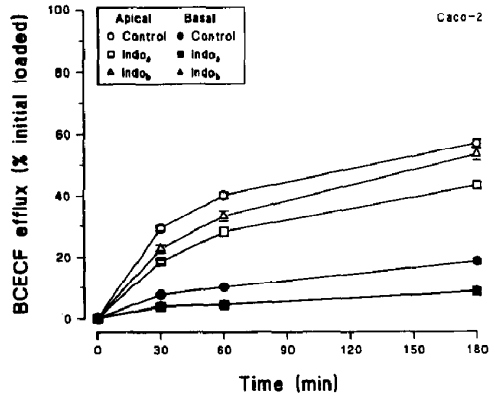


Fig. 7. Polarity of BCECF efflux from Caco-2 epithelial layers as a function of time. Caco-2 cells were loaded with BCECF and then transferred to serum-free medium or serum-free medium containing indomethacin 0.5 mM in either the apical or basal solutions. BCECF efflux is expressed as a percentage of the total initial BCECF in the layers. Results are illustrated as means ± 1 SE (N = 8).

BCECF efflux from epithelial layers of another human intestinal cell line, HCT-8, was not polarized (Fig. 8). Basal efflux of BCECF was markedly and equally reduced by apical and basal addition of indomethacin. In contrast, apical efflux of BCECF was less sensitive to inhibition by indomethacin being slightly, but not significantly, more sensitive to basal indomethacin (Fig. 8). Yet a different pattern was observed with a third human intestinal epithelial cell line, $T_{84}$. Similar to the results with the HCT-8 layers, BCECF efflux over 5 hr from $T_{84}$ layers was equivalent into the apical [43 ± 1% (N = 4)] and basal solutions [46 ± 1% (N = 4)]. Indomethacin, 0.5 mM, added to basal solution markedly reduced both apical and basal efflux of BCECF [2 ± 1% and 9 ± 2% (N = 3), respectively]. In contrast, addition of indomethacin to the apical solution was far less effective in inhibiting either apical or basal BCECF efflux [32 ± 1% and 33 ± 5% (N = 3)], respectively.

*Effect of indomethacin on transepithelial vinblastine secretion*

We have demonstrated previously that epithelial layers formed from MDCK, HCT-8 and $T_{84}$ cells demonstrate vectorial secretion of vinblastine, consistent with functional polarized expression of P-glycoprotein [10, 11]. We investigated the effect of indomethacin on vinblastine secretion. In MDCK epithelial layers there was a net secretory flux ($J_{net}$) of vinblastine, i.e. vinblastine flux in the basal-to-apical direction ($J_{b-a}$) exceeded flux in the apical-to-basal direction ($J_{a-b}$); apparent vinblastine permeability 1.71 ± 0.11 and 0.59 ± 0.18 × $10^{-5}$ $cm^2$/hr (N = 3), respectively (Fig. 9). Nifedipine, 50 μM, reduced $J_{b-a}$ to values similar to that of $J_{a-b}$ (Fig. 9) such that there was no longer a net secretory flux; apparent vinblastine permeability 1.05 ± 0.08 and 0.93 ± 0.13 × $10^{-5}$ $cm^2$/hr (N = 6), respectively. The

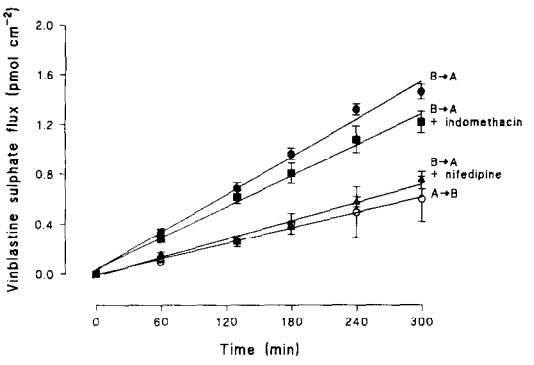


Fig. 9. Transepithelial vinblastine sulphate fluxes in MDCK epithelial layers. [$^3$H]Vinblastine flux is illustrated in the basolateral-to-apical (B–A) and apical-to-basolateral (A–B) under control conditions (N = 3), and in the basolateral-to-apical direction in the presence of 0.5 mM indomethacin (N = 9) or 50 $\mu$M nifedipine (N = 6), added to both apical basal bathing solutions. The lines illustrate the least-squares best fit to the data, the slopes of which give the vinblastine flux rate. Data are illustrated as mean values ± SE.

slight increase in $J_{a-b}$ observed with nifedipine is correlated with a reduction in transepithelial electrical resistance, as has been reported previously with verapamil [10]. In contrast to the inhibition of $J_{net}$ with nifedipine, indomethacin, 0.5 mM, had no significant effect on vinblastine secretion by MDCK cell layers (Fig. 9); apparent vinblastine permeability 1.43 ± 0.08 and 0.55 ± 0.05 × $10^{-5}$ cm$^2$/hr (N = 9), respectively.

Similar results were observed in HCT-8 cell layers. $J_{b-a}$ exceeds $J_{a-b}$ for vinblastine in HCT-8 cells, as has been observed previously [10], although $J_{net}$ in HCT-8 cell layers is less than that observed for MDCK cell layers; apparent vinblastine permeability 1.77 ± 0.10 and 1.06 ± 0.08 × $10^{-5}$ cm$^2$/hr (N = 3), respectively. Indomethacin, 0.5 mM, increased $J_{b-a}$ [apparent permeability 2.24 ± 0.10 × $10^{-5}$ cm$^2$/hr (N = 3)], concomitant with a reduction in transepithelial electrical resistance.

## DISCUSSION

The polar nature of BCECF efflux in renal MDCK and intestinal Caco-2 epithelial cell layers is consistent with its transport by a cellular secretory detoxifying mechanism in the kidney and intestine. In MDCK cells, where detailed morphometric studies have been carried out [17], the basolateral membrane surface area is at least 7-fold greater than the apical area. Thus, the polarity of BCECF efflux cannot be explained in terms of passive leak through membranes of differing surface area. In contrast, the ATP-dependent nature of BCECF efflux [2, 4] points to a carrier-mediated transport process. This conclusion is supported by the unique pharmacological profile for inhibition of BCECF efflux [4]. ATP-dependent carrier-mediated efflux of BCECF has been described recently in a prokaryote, *Lactococcus lactis* [18, 19].

The pharmacological profile for inhibition of BCECF efflux is, at least qualitatively, similar in a range of epithelial cells. Thus, indomethacin is an effective inhibitor of BCECF efflux in all epithelial cells investigated, while DIDS and SITS are relatively ineffective agents. Other non-steroidal anti-inflammatory drugs, in addition to indomethacin, also reduce BCECF efflux [3], but the concentrations of this class of drugs required to reduce BCECF efflux are greater than those required to inhibit cyclooxygenase activity [14],while the inhibition of BCECF efflux with indomethacin is unaffected by exogenous prostaglandins [4], indicating that inhibition of cyclooxygenase activity is unlikely to be involved in the mechanism of action. Another inhibitor of anion channels, NPPB, was more effective an inhibitor of BCECF efflux than indomethacin in MDCK cells, which contrasts with a lower activity in HCT-8 cells [4]. Indomethacin [20] and NPPB [21] are both agents which interfere with anion transport. Replacement of external $Cl^-$ with a variety of anions only resulted in a small reduction in BCECF efflux with the impermeant methanesulphonate, suggesting that BCECF efflux is unlikely to be mediated by an anion exchange mechanism. NPPB is a $Cl^-$ channel blocker [21] and inhibits adrenaline-stimulated $Cl^-$ secretion in MDCK cell layers [22]. BCECF efflux was unaffected by the addition of adrenaline, providing no evidence for the involvement of secretory $Cl^-$ channels in its efflux pathway. The indomethacin-insensitive component of BCECF efflux will include an undefined proportion of passive leak of the fluorochrome.

We have exploited the inhibitory activity of indomethacin on BCECF efflux to further characterize the polarized nature of the efflux mechanisms. Indomethacin reduced BCECF efflux, but the relative distribution, apical or basolateral, of the indomethacin-sensitive component differed amongst the five cell lines investigated. The effectiveness of indomethacin, applied to either the apical or basal surfaces of the cell layers, also varied with the different cell lines. In contrast, the relative distributions of the indomethacin- and NPPB-sensitive components of BCECF efflux in Strain I MDCK cells were similar. The factors regulating the polarity of BCECF efflux, both the indomethacin-sensitive and -insensitive components, have yet to be elucidated.

We have reported previously that a number of the epithelial cell layers used in the present study demonstrate vectorial secretion of vinblastine, indicating polarized functional expression of P-glycoprotein [10, 11]. P-glycoprotein belongs to the ATP-binding cassette super-family of membrane transport proteins, which includes numerous bacterial, yeast and insect import and export pumps, the mammalian cystic fibrosis transmembrane regulator, the major histocompatibility-linked peptide transporter and the *MDR3* product of unknown function [23–25]. Other fluorochromes, including fluo-3 used as an intracellular $Ca^{2+}$-indicator, are reported to be substrates for P-glycoprotein [6–8].

BCECF efflux is reduced by agents such as vinblastine and actinomycin D which are recognised to interact with P-glycoprotein [2, 4]. However, other pharmacological agents that are potent inhibitors of P-glycoprotein-mediated vinblastine secretion, such as nifedipine and reserpine [4], are ineffective against BCECF efflux. Similarly, vinblastine secretion was unaffected by indomethacin, a potent inhibitor of BCECF efflux. Thus, BCECF efflux cannot be equated with P-glycoprotein function. A similar conclusion has been arrived at for the ATP-dependent efflux of BCECF from *L. lactis* [19]. It is interesting to speculate that BCECF efflux may be mediated by an indomethacin-sensitive ATP-binding cassette transporter protein. The relationship between BCECF efflux mechanisms in the eukaryotic and prokaryotic systems awaits the availability of molecular tools for investigation.

Polarized efflux of an ion-sensitive fluorochrome such as BCECF has implications for studies of cytosolic dye concentrations using single cell image analysis techniques [26–28]. Local depletion of fluorochrome due to activation of efflux mechanisms might give rise to reduced fluorescent signals which could be erroneously interpreted as indicating local changes in intracellular pH. In addition, sequestration of fluorochromes into intracellular organelles might also contribute to similar spurious interpretations of fluorescent signals.

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