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Efflux of bis-carboxyethyl-carboxyfluorescein (BCECF) by a novel ATP-dependent transport mechanism in epithelial cells

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The efflux of the intracellular pH fluorochrome 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was quantified in four cultured epithelial cell lines; HCT-8, T84, HGT-1 and MDCK. BCECF efflux was time-dependent, and after 5 h 45-91% of the initial BCECF loaded was extracellular, efflux being greatest in MDCK cells. Depletion of cellular ATP approximately halved BCECF efflux. BCECF efflux was inhibited by indomethacin, vinblastine and verapamil, but not by nifedipine or reserpine. Certain features of BCECF efflux resemble drug efflux in multidrug resistant cells, but inhibition of efflux displays a distinct pharmacological profile suggesting BCECF is a substrate for a novel ATP-dependent transport system.

Considerable recent interest has been shown in the diverse superfamily of ATP-dependent transport proteins [1-4], which include the mammalian *mdr1* gene product, P-glycoprotein, implicated as a drug efflux pump in multidrug resistance (mdr) [2], the *pfmdr* gene product associated with chloroquine-resistance in *Plasmodium falciparum* [5], and the cystic fibrosis transmembrane regulator (CFTR) protein, of unknown function [6]. Drug efflux in cells expressing *mdr1* has a well-recognised pharmacological profile, including the ability of verapamil, nifedipine and reserpine to inhibit this efflux, so reversing the mdr phenotype [7-9].

The fluorochrome 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) is widely used as an indicator of intracellular pH, and dye loss has been noted as a limiting factor in such experiments [10]. This has necessitated the use, in one report, of an anion exchange resin to act as a sink to bind the extracellular BCECF [11]. In the present study we report that BCECF efflux from cultured epithelial cells is an energy-dependent process resembling drug efflux in cells expressing *mdr1*. In addition, inhibition of BCECF efflux by a number of

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pharmacological agents shows that the transport system mediating BCECF efflux is distinct from mdr.

Materials and Methods

Human ileal-caecal (HCT-8), colonic (T84) and gastric (HGT-1) adenocarcinoma, and Madin-Darby canine kidney (MDCK) epithelial cells were seeded into 24 well plates at 10^5-$5x10^5$ cells/well and allowed to form confluent monolayers. Cells were pre-loaded with BCECF by addition of the non-fluorescent acetoxymethyl ester form of BCECF (BCECF-AM, Molecular Probes Inc.: HCT-8 and HGT-1, 20 μg ml^{-1}; T84 and MDCK, 40 μg ml^{-1}) for 70 min at 20°C. The cells were then washed twice with 1 ml of phosphate buffered saline (PBS) before efflux was studied at 37°C and 5% CO_2 in the appropriate serum-free cell culture medium containing glucose for each cell line. At pre-determined intervals, the cell culture medium was removed for analysis, and replaced with fresh medium. BCECF fluorescence was quantified, after diluting the cell culture medium 1:100 in PBS, at 538 nm emission and 485 nm excitation, in a Perkin-Elmer LS-5 spectrofluorimeter. At the end of the experiment, remaining intracellular BCECF was measured after the cells were lysed by the addition of 0.1% (v/v) Triton X-100. The rate of efflux of BCECF was calculated as a percentage of the total initial BCECF fluorescence.

In order to deplete cellular ATP pools, 15 mM sodium azide and 50 mM 2-deoxy-D-glucose (2-DOG) were added to the serum-free medium for the entire incubation [8]. The effects of indomethacin, verapamil, nifedipine, reserpine and vinblastine sulphate were investigated by their presence throughout the measurement of BCECF efflux. None of the drugs interfered with measurement of BCECF fluorescence (data not shown). BCECF efflux was expressed as a percent of BCECF efflux under control conditions, in the presence of serum-free medium alone. Significance of difference between groups was tested by Student's *t* test, with significance set at $P < 0.05$. Data is expressed as the mean ± 1 S.E.M.

Results

BCECF efflux was observed in all four epithelial cell lines investigated. The efflux was time-dependent, and after 5 h was 76 ± 6 (n=41), 77 ± 2 (n=7), 91 ± 0 (n=3) and 45 ± 4 (n=11) % of the original BCECF loaded into HCT-8 (Fig. 1), T84, MDCK and HGT-1 cells, respectively, was extracellular. In HCT-8 cells the rate of BCECF efflux was lower during the first hour, and the fractional rate of BCECF efflux [12] was still greater at 4-5 h, 0.142 ± 0.011 h^{-1} (n=4), as compared with 0-1 h, 0.051 ± 0.003 h^{-1} (n=4) (Fig.1). Replacement of medium NaCl by KCl to depolarise the transmembrane electrical p.d. in HCT-8 cells had no effect upon BCECF efflux into the medium (control efflux was 64±2% of total fluorescence, efflux in high K was 65±3% of total fluorescence at 5h, n=6). Inclusion of 10μM or 100μM BCECF in the extracellular incubation medium had no effect upon the amount of BCECF remaining within HCT-8 cells. Control homogenates had 18±3% (n=6) of the total fluorescence measurable still in the monolayers after 5h, homogenates exposed to external BCECF had 16±2% and 14±2% (n=7) remaining for external concentrations of 10 and 100μM respectively. Therefore BCECF efflux is not simply dependent upon the prevailing transmembrane electrochemical gradient. Sodium azide and 2-deoxy-D-glucose treatment in HCT-8 cells was associated

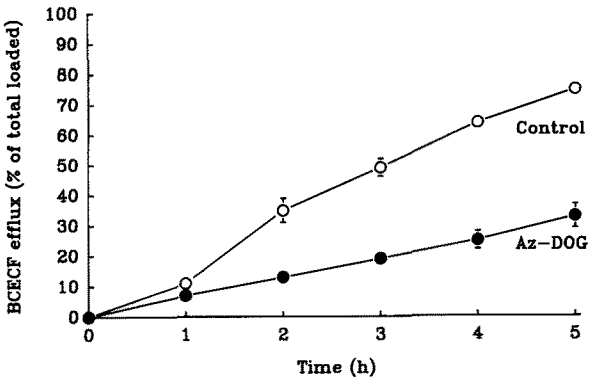

Fig 1. Energy dependence of BCECF efflux in HCT-8 cells with time. BCECF efflux was measured at 37°C in serum free medium containing glucose (control, ○), in the presence of 15 mM sodium azide and 50 mM 2-deoxy-D-glucose (Az-DOG; ●) over 5 h. Results are plotted as mean ± 1 s.e. (n=4) BCECF efflux (expressed as a percent of the total initial BCECF).

with a substantial loss of intracellular K (control cells 170 ± 10 μmoles/10^6 cells, n=8, with sodium azide/2-DOG treatment intracellular K was below detection limits consistent with the abolition of Na-K-ATPase activity). In contrast to the enhanced K loss, sodium azide and 2-deoxy-D-glucose treatment reduced BCECF efflux at all time points (Fig. 1), and at 5 h BCECF efflux was 44 ± 5 (n=4) and 44 ± 1 (n=4) % of control values in HCT-8 (Fig. 1) and T84 cells, respectively.

Table 1. Inhibition of BCECF efflux from epithelial cells

Inhibitor	Conc. (μM)	BCECF efflux (% of control efflux)			
		HCT-8	T84	MDCK	HGT-1
Indomethacin	500	21±2 (30)*	50±3 (6)*	52±3 (3)*	21±6 (6)
Vinblastine	100	41±8 (6)*	45±3 (3)*	45±3 (3)*	N.D.
Verapamil	50	64±11 (3)*	97±3 (3)	86±2 (3)	N.D.
Nifedipine	100	95±3 (3)	N.D.	99±1 (3)	N.D.
Reserpine	100	96±3 (3)	98±1 (3)	98±2 (3)	N.D.

Results are expressed as mean ± 1 s.e. (observations) BCECF efflux expressed as a percent of efflux in control cells without inhibitor (5h).

* BCECF efflux significantly reduced as compared with control efflux.

ND (not determined).

BCECF efflux was modulated by a variety of pharmacological agents. Indomethacin, 500 μM, reduced BCECF efflux to 21-52 % of control in all four cell lines (Table 1), with IC_{50} values (concentration required to produce 50% of maximal inhibition) of 103.8 ± 0.7, 97.6 ± 0.9 and 23.6 ± 0.3 μM in HCT-8, T84 and HGT-1 cells, respectively. The P-glycoprotein substrate vinblastine sulphate, 100 μM, resulted in greater than 50 % inhibition of BCECF efflux in HCT-8, T84 and MDCK cells (Table 1), with an IC_{50} in HCT-8 cells of 4 μM. Verapamil, 50 μM, a calcium channel antagonist which inhibits P-glycoprotein-mediated drug efflux at 1-2 μM [8], reduced BCECF efflux in HCT-8 cells, but was less effective in MDCK, and ineffective in T84 cells (Table 1). Nifedipine, another calcium channel antagonist which is an inhibitor of P-glycoprotein at a concentration of 50 μM [8], was unable to reduce BCECF efflux at concentrations ranging from 1 to 1000 μM (Table 1). A third drug with the ability to inhibit P-glycoprotein, reserpine [8] was also ineffective, at concentrations of 1 to 1000 μM, as an inhibitor of BCECF efflux (Table 1).

Discussion

The present study demonstrates that cellular efflux of BCECF is by an ATP-dependent mechanism with a novel pharmacological profile. The efflux of several other fluorochromes has been related to activity of P-glycoprotein, and was sensitive to known inhibitors of P-glycoprotein, including verapamil and reserpine [12,13]. Both HCT-8 and T84 express *mdr1* [15; Hunter, Simmons & Hirst, unpublished]. Although verapamil was able to interfere with BCECF efflux in at least one cell line, two other antagonists of P-glycoprotein-mediated drug efflux, reserpine and nifedipine, were unable to reduce BCECF efflux even at concentrations much greater than those required to inhibit P-glycoprotein activity [7-9]. In contrast, indomethacin, an agent which has not been associated with reversal of mdr or inhibition of P-glycoprotein mediated drug efflux, was a potent inhibitor BCECF efflux. In addition, several common inhibitors of anion transport systems and anion conductances, e.g., furosemide, 4-acetamid-4'-isocyanatostilbene-2,2'-disulfonic acid (SITS), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), probenecid and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), were ineffective inhibitors of BCECF efflux at concentrations giving maximal anion transport inhibition (manuscript in preparation). Thus the pharmacological profile for inhibition of BCECF efflux is indicative of a novel mechanism. Nevertheless, vinblastine sulphate, a mdr substrate, was a potent inhibitor of BCECF efflux. This latter observation, considered with the inhibitory action of verapamil and the energy-dependent nature of BCECF efflux, is suggestive of its mediation by a novel ATP-dependent efflux mechanism related to the superfamily of membrane transport proteins [1-4].

We have noted a reduced initial efflux rate of BCECF in control cells (Fig. 1). This may result from intracellular acid generation during release of BCECF by esterase activity from the BCECF-AM. However, fluorescent ratio intensity measurements of BCECF-loaded HCT-8 cells to determine intracellular pH [16] gave values of 7.0-7.2 (unpublished observations). An alternative hypothesis could involve intracellular endosomal dye accumulation followed by translocation and exocytosis at the plasmalemma. Such a mechanism has been suggested for mdr [17]. However, accumulation of BCECF in intracellular organelles is reported to be low [10,18], and direct visualisation of BCECF-loaded HCT-8 cells under epi-fluorescence revealed an uniform distribution of the fluorochrome.

BCECF carries four negatively charged carboxyl groups, this molecular configuration designed to entrap it within the cytosol of cells after hydrolysis of the uncharged ester form [10]. P-glycoprotein substrates are lipophilic cations, and the pharmacophore contains a basic nitrogen atom and two planar aromatic rings [19]. BCECF may be a model substrate for a related ATP-dependent transport protein with a specificity for anions. There are already possible candidates for this protein, including the gene product of *mdr3* [20], and CFTR [6], both with unknown physiological functions, and both members of the superfamily of ATP-dependent transport proteins [1-4]. Of the cell lines studied here, at least T84 cells express CFTR [6].

In addition to its well-defined role in mediating multidrug resistance in cancer cells, the physiological role of P-glycoprotein in epithelial cells of tissues such as the intestine and kidney has been proposed as detoxification mechanism [21]. The novel energy-dependent efflux system reported here may similarly act to excrete, or limit absorption of another class of xenobiotics.

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