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PHARMACOLOGICAL PROFILE OF INHIBITION OF 2′,7′-BIS(2-CARBOXYETHYL)-5(6)-CARBOXYFLUORESCEIN EFFLUX IN HUMAN HCT-8 INTESTINAL EPITHELIAL CELLS

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Abstract—The efflux of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) from human HCT-8 intestinal epithelial cultured cells was time-dependent, and after 5 hr 76% of the fluorochrome was extracellular. The pharmacological profile for inhibition of this efflux has been investigated, focusing on agents which modulate anion transport. BCECF efflux was sensitive to inhibition by 0.5 mM indomethacin (50% inhibition at 20 μM) which reduced efflux to values observed after depletion of ATP with azide and 2-deoxy-D-glucose. Indomethacin inhibition of BCECF efflux was not reversed with prostaglandin. The stilbene derivatives 4-acetamido-4′-isothiocyano-2-2′-disulphonic stilbene and 4,4′-diisothiocyano-2,2′-disulphonic stilbene only resulted in partial inhibition of BCECF efflux, even at 1 mM. Furosemide, bumetamide, probenecid and 5-nitro-2-(3-phenylpropyl-amino)-benzoate only reduced BCECF efflux at 1 mM. The cationic agent vinblastine was as active as indomethacin as an inhibitor of BCECF efflux (50% inhibition with 10 μM) while actinomycin D was also a good inhibitor (50% inhibition with 100 μM). Several other cationic agents, including nifedipine, amiloride and reserpine, were ineffective as inhibitors of BCECF efflux in concentrations up to 1 mM. Thus, the pharmacological profile for inhibition of BCECF efflux does not fully equate with any recognised transport system. Agents such as cytochalasin B and chloroquine did not effect BCECF efflux suggesting accumulation and subsequent discharge from endosomes is not a pathway for secretion. BCECF may be a substrate for a cellular secretory detoxifying system in epithelial cells.

Carboxy analogues of fluorescein are widely used as indicators of intracellular pH. The use of carboxyfluorescein has been limited by the marked dye loss from cells which led to the development of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF†). BCECF loss from cells, although still significant, is markedly reduced arguably due to its lower lipophilicity as a consequence of the greater number of charged carboxy groups [1]. The presumed intracellular trapping of BCECF has led recently to its increased use as an impermeant marker whose loss has been suggested to be correlated with cytolysis [2, 3]. Each role will be limited by dye transport from cells. We have reported recently that BCECF loss from a variety of epithelial cells, including human intestinal adenocarcinoma HCT-8 and T84 cells, is appreciable and is not simply passive leakage but may be mediated via an ATP-dependent transport mechanism [4, 5]. In the present study we have characterized the pharmacological profile for inhibition of BCECF efflux in HCT-8 cells from the medium, focusing on modulators of anion transport systems.

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† Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; NPPB, 5-nitro-2-(3-phenylpropyl-amino)-benzoate, SITS, 4-acetamido-4′-isothiocyano-2-2′-disulphonic stilbene; DIDS, 4,4′-diisothiocyano-2,2′-disulphonic stilbene; PBS, phosphate-buffered saline; NSAID, non-steroidal anti-inflammatory drugs.

MATERIALS AND METHODS

Cell culture. HCT-8 cells [6] were obtained from the American Type Culture Collection and maintained in RPMI 1640 with 10% horse serum, 1 mM glutamine and 1 mM sodium pyruvate at 37° in 5% CO_2/95% air. Confluent monolayers were subcultured by treatment with 0.05% trypsin and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free PBS. Cells were seeded into 24-well plates at a density of 1.5×10^5 cells/well and allowed to form monolayers over the next 2–4 days. All cell culture media and supplements (Gibco BRL) and plastic cell culture flasks and plates (Nunc) were purchased from Life Technologies (Paisley, U.K.).

BCECF efflux. Cell monolayers were washed twice with 1 mL PBS before loading with BCECF by the addition of the non-fluorescent cell-permanent acetoxymethyl ester form of BCECF [BCECF-AM (Molecular Probes, OK, U.S.A.), 20 μg/mL] for 70 min at 20°C. The cells were then washed twice with 1 mL PBS, and efflux was studied over 5 hr at 37° and 5% CO_2, in serum-free medium with and without test substances. The medium was removed and the cells were lysed by addition of 0.1% Triton X-100. BCECF was quantified, after diluting the samples 1:100 in PBS, by fluorescence (excitation,

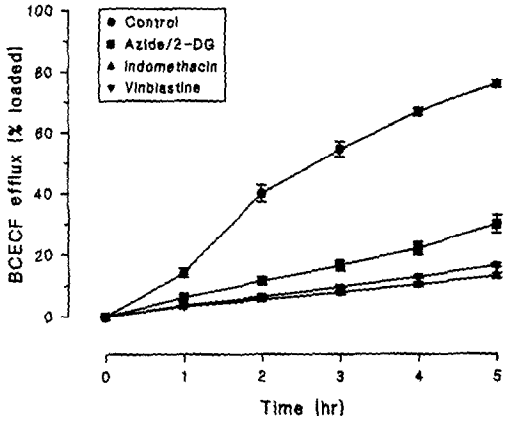

Fig. 1. BCECF efflux from HCT-8 cells as a function of time. HCT-8 cells were loaded with BCECF and then transferred to serum-free medium (N = 8) or medium containing 15 mM sodium azide, and 50 mM 2-deoxy-D-glucose (Azide/2-DG; N = 8), 0.5 mM indomethacin (N = 4) or 0.1 mM vinblastine (N = 4). BCECF efflux is expressed as percentage of the total initial BCECF in the cells. Results are expressed as means ± 1 SE.

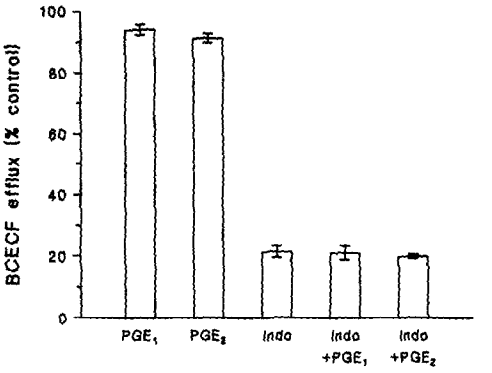

Fig. 2. Effect of 10^{-7} M prostaglandin E_1 or E_2 on BCECF efflux over 5 hr, in the absence and presence of 0.5 mM indomethacin. Indomethacin (N = 27) reduced significantly BCECF efflux while neither PGE_1 (N = 4) or PGE_2 (N = 8) altered significantly control rate of efflux, or efflux reduced by indomethacin. Results are expressed as mean BCECF efflux expressed as percentage of control efflux ± 1 SE.

485 nm; emission, 538 nm) in a Perkin–Elmer LS-5 spectrofluorimeter. 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. BCECF efflux was expressed as a percentage of total cellular BCECF.

Effect of pharmacological agents. Stock solutions of agents were freshly prepared in serum-free medium or 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 Molecular Probes or Life Technologies. Pharmacological agents were supplied by the Sigma Chemical Co. (Poole, U.K.) except vinblastine sulphate (Lederle, Wayne, NJ, or Lilly), actinomycin D (Merck Sharp & Dohme) and methotrexate (David Bull Labs). NPPB was a gift from Smith Kline Beecham Pharmaceuticals (Welwyn, U.K.).

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

RESULTS

BCECF efflux from HCT-8 cells was a time-dependent process and after 5 hr 76 ± 2% (N = 41) of the fluorochrome had been released into the medium (Fig. 1). In cells depleted of ATP by addition of 15 mM sodium azide and 50 mM 2-deoxy-D-glucose [4, 7], the rate of BCECF efflux was considerably retarded being only 37 ± 4% (N = 8) of control efflux at 5 hr (Fig. 1).

Indomethacin, in addition to its well recognised

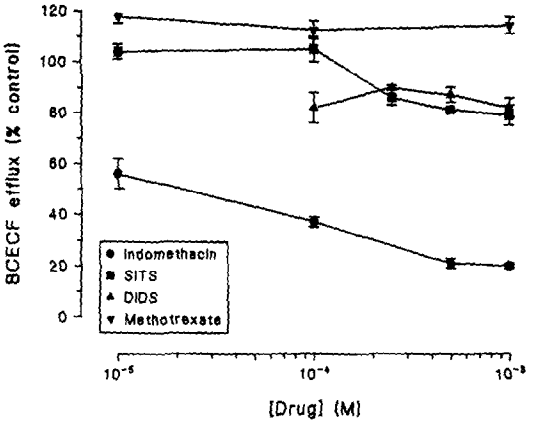

Fig. 3. Concentration–response relations for inhibition of BCECF efflux over 5 hr by indomethacin, SITS, DIDS and methotrexate. Results are expressed as mean BCECF efflux expressed as percentage of control efflux ± 1 SE (N = 6).

inhibition of cyclo-oxygenase, has been noted previously as a potent inhibitor of BCECF efflux [4, 5]. The effect of 0.5 mM indomethacin on the time-course of BCECF efflux is illustrated in Fig. 1. Indomethacin reduced BCECF efflux to values lower than those observed with ATP depletion. Exogenous prostaglandins (0.1 μM PGE_1 or PGE_2) did not significantly modify BCECF efflux, nor were they able to ameliorate the inhibitory activity of 0.5 mM indomethacin upon BCECF efflux (Fig. 2).

The concentration of indomethacin required for 50% inhibition of BCECF efflux was 20 μM (Fig. 3). After 5 hr, BCECF efflux was reduced to 56 ± 6% (N = 6) of the control rate with 10 μM indomethacin. Maximal inhibition was observed at 0.5 mM indomethacin [20 ± 2% (N = 31)], the same

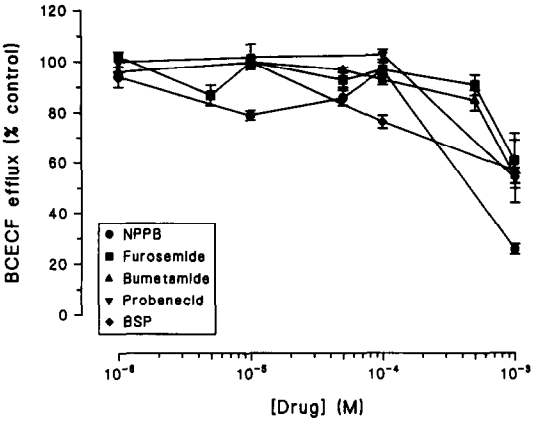

Fig. 4. Concentration–response relations for inhibition of BCECF efflux over 5 hr by NPPB, furosemide, bumetamide, probenecid and bromosulphophthalein (BSP). Results are expressed as mean BCECF efflux expressed as percentage of control efflux ± 1 SE (N = 6).

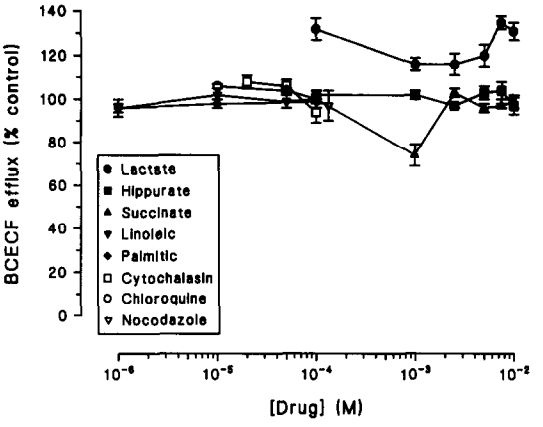

Fig. 5. Concentration–response relations for inhibition of BCECF efflux over 5 hr by lactate, hippurate, succinate, linoleic acid, palmitic acid, cytochalasin B, chloroquine and nocodazole. Results are expressed as mean BCECF efflux expressed as percentage of control efflux ± 1 SE (N = 6).

reduction as with 1 mM indomethacin [20 ± 1% (N = 6)] (Fig. 3). At concentrations of indomethacin > 1 mM indomethacin had a reduced effect on BCECF efflux and by 10 mM the efflux had returned to control values [5]. The reversal of the inhibitory action of indomethacin on BCECF is a result of acute toxicity and is correlated with increased leak of lactate dehydrogenase and ^{51}Cr [5].

The stilbene derivatives SITS and DIDS, inhibitors of Cl^-/HCO_3^--exchange [8, 9], had only moderate inhibitory activity on BCECF efflux compared to indomethacin. SITS did not affect BCECF efflux at concentrations ≤0.1 mM and only reduced efflux to 79 ± 4% (N = 3) of control values at 1 mM (Fig. 3). DIDS also moderately reduced BCECF efflux, the reduction being ~20% with concentrations of 0.1–1 mM (Fig. 3). Methotrexate 10 nM–1 mM did not reduce BCECF efflux and at ≥10 μM resulted in an enhanced rate of BCECF efflux, approximately 120% of control (Fig. 3).

The Cl^- channel antagonist NPPB [10] was more effective in reducing BCECF efflux although concentrations >0.1 mM were required (Fig. 4). The loop diuretics furosemide and bumetamide are potent inhibitors of $Na^+/K^+/2Cl^-$ co-transport [11] but were capable of significant reductions in BCECF efflux only when applied at 1 mM but not at lower concentrations (Fig. 4). Probenecid, an inhibitor of organic anion exchange [8], resulted in ~50% inhibition of the control rate of BCECF efflux at a concentration of 1 mM (Fig. 4). Bromosulphophthalein, a substrate for organic anion transport [8], resulted in a concentration-dependent inhibition of BCECF efflux causing approximately 25 and 50% inhibition at 0.1 and 1 mM, respectively (Fig. 4). Acetate, succinate and hippurate, in concentrations up to and including 10 mM did not reduce BCECF efflux (Fig. 5). At the concentrations investigated, 0.1–10 mM, lactate enhanced BCECF efflux to 120–130% of control. Neither a *cis*, linoleic, nor a *trans*, palmitic, unsaturated fatty acid influenced BCECF efflux over 5 hr at concentrations of 1–100 μM (Fig. 5).

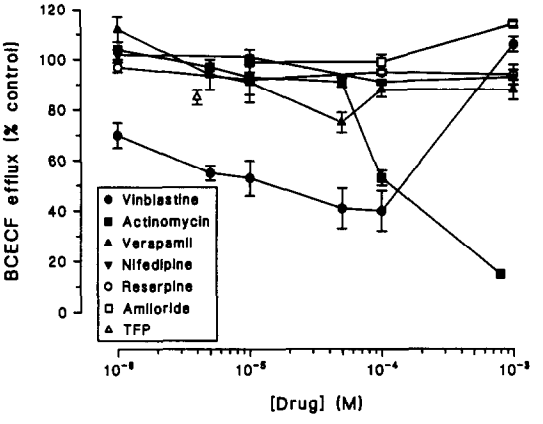

Fig. 6. Concentration–response relations for inhibition of BCECF efflux over 5 hr by vinblastine, actinomycin D, verapamil, nifedipine, reserpine, amiloride and trifluoperazine (TFP). Results are expressed as mean BCECF efflux expressed as percentage of control efflux ± 1 SE (N = 6).

The cationic cytotoxic agent vinblastine was tested; surprisingly, marked reductions in BCECF efflux were noted comparable to that seen with indomethacin (Fig. 1). Vinblastine resulted in concentration-related reductions in BCECF efflux when applied at concentrations varying from 1 to 100 μM, with 50% inhibition at 10 μM (Fig. 6). At a higher concentration of 1 mM, BCECF efflux returned to control values associated with visible disruption of the cell monolayer, presumably as a result of acute membrane damage and enhanced permeability to BCECF as has been noted previously with high concentrations of indomethacin [5]. BCECF efflux

was less sensitive to inhibition by a second cytotoxic agent actinomycin D but concentrations of 0.1 and 1 mM still resulted in a marked inhibition of efflux, and 50% inhibition at 100 μM (Fig. 6). Both actinomycin D and vinblastine are substrates for P-glycoprotein [12].

Five agents, the Ca^{2+}-channel antagonists verapamil and nifedipine, the calmodulin antagonist trifluoperazine, the Na^+-channel and Na^+/H^+ exchange inhibitor amiloride, and the indole alkaloid reserpine, recognised for their ability to interfere with cation transport processes and also able to reverse the multidrug resistance phenotype by inhibiting the activity of P-glycoprotein [13, 14] were relatively ineffective at reducing BCECF efflux (Fig. 6). Amiloride, reserpine and nifedipine did not reduce efflux significantly while verapamil resulted in a significant inhibition only at a concentration of 50 μM [25 ± 4% (N = 6) inhibition]. A small reduction (15%) was observed with 4 μM trifluoperazine (Fig. 6).

Agents used to interfere with endosomal and/or lysosomal function [13] were ineffective against BCECF efflux. Neither the lysosomotropic agent chloroquine nor cytochalasin B, a drug that disrupts microfilaments, affected BCECF efflux in concentrations up to 100 μM. Nocodazole 133 μM, an agent which disrupts microtubules [15], did not affect BCECF efflux (Fig. 5).

DISCUSSION

In HCT-8 cells preloaded with BCECF, using the acetoxymethyl ester derivative, appreciable amounts are lost to the bathing medium; that this loss represents predominately transport-mediated flux is evident by the inhibitory action of a number of agents including indomethacin. The use of BCECF as an impermeant membrane probe to monitor cytolysis is, therefore, invalidated unless appropriate controls are included [5]. The use of BCECF over long time periods to monitor intracellular pH in cells will also be limited by dye transport. There is considerable interest in pharmacological agents that may limit such loss. Clearly the identity of the transporter mediating BCECF efflux is crucial; it would be unwise to use agents that will potentially inhibit transport systems involved in pH regulation, e.g. Cl^-/HCO_3^- exchange. BCECF efflux was reduced by ATP depletion. Whether the efflux is mediated by a transport process directly coupled to ATP utilization or whether the general cellular disturbances caused by ATP depletion indirectly result in a reduction in BCECF efflux requires further investigation. The greater inhibitory effect of indomethacin or vinblastine on BCECF efflux as compared to the azide/deoxy-D-glucose treatment may be an indication of the latter only causing partial depletion of ATP pools, that the treatment itself increases passive leakage of BCECF or that a component of BCECF efflux is ATP-independent.

Indomethacin was a potent inhibitor of BCECF efflux and inhibition did not appear to be modulated by inhibition of cyclo-oxygenase as it was not reversed by exogenous prostaglandin. Two other NSAID, sulindac (10 μM–10 mM) and ketoprofen (1–10 mM) but not aspirin (10 μM–10 mM), also inhibit BCECF efflux [5]. Higher concentrations of indomethacin (>1 mM) are associated with a progressive reduction of inhibitory activity against BCECF efflux [5]. This loss of inhibition of BCECF efflux is correlated with increased leak of LDH and ^{51}Cr from the cells, consistent with indomethacin-induced cell lysis [5]. These high concentrations, >1 mM, of indomethacin are also toxic as assessed by the ability of the cells to reduce a tetrazolium dye using mitochondrial dehydrogenase enzymes (MTT assay) and to accumulate the supravital dye Neutral red [6]. Concentrations of NSAID required to reduct MTT activity are considerably greater than those required to reduce BCECF efflux [5, 6] and thus the NSAID-induced inhibition of BCECF efflux cannot be explained by uncoupling of ATP production. Recent reports point to indomethacin being a potent inhibitor of anion exchange [16], and non-erythroid anion exchangers are relatively resistant to inhibition by DIDS [9]. It should be noted that BCECF efflux is only partially inhibited by either SITS or DIDS even at 1 mM.

BCECF efflux displays at least a passing similarity to a variety of other cellular transport systems for xenobiotics, particularly transport systems of organic anions. Methotrexate efflux from a variety of cells is via an ATP-dependent mechanism which is sensitive to inhibition by bromosulphophthalein, probenecid and verapamil [17, 18]. Although probenecid and bromosulphophthalein inhibited BCECF efflux, verapamil had a slight inhibitory effect only, being optimal at 50 μM. Methotrexate enhanced BCECF efflux at concentrations of 0.01 to 1 mM, presumably a reflection of its toxicity. The efflux of another anionic fluorochrome, the calcium indicator fura-2, is also sensitive to probenecid, at least when used at concentrations >1 mM [19]. Organic anion transport in the kidney and choroid plexus is also sensitive to inhibition with probenecid and hippurate, as well as SITS, DIDS and furosemide [8, 20]. These agents only had a moderate, if any, effect on BCECF efflux. The Cl^- channel antagonist NPPB [10] did not influence BCECF efflux at concentrations ≤100 μM. Nevertheless, NPPB did inhibit BCECF efflux at 1 mM, a concentration well above that commonly used to inhibit Cl^- transport. Inhibition of BCECF efflux by high concentrations of agents such as NPPB may be a consequence of non-specific mechanisms resulting in ATP depletion. *Cis*, but not *trans*, unsaturated fatty acids have been shown to inhibit directly Cl^--channels and to inhibit Cl^--mediated short-circuit current in colonic epithelial cells at concentrations of 10–50 μM [21], but neither lineolic or palmitic, examples of *cis* and *trans* unsaturated fatty acids, affected BCECF efflux. BCECF efflux was also unaffected by cellular depolarization [4].

In addition to a marked sensitivity to inhibition with the non-steroidal anti-inflammatory drug indomethacin, two cytotoxic agents, vinblastine and actinomycin D, were also effective inhibitors of BCECF efflux. Intracellular concentrations of a variety of cytotoxic compounds, generally cationic and including vinblastine and actinomycin D, are reduced by the ATP-dependent efflux modulated

by P-glycoprotein [12]. HCT-8 cells express P-glycoprotein and in these cells vinblastine efflux is inhibited by verapamil [22], an agent which is a competitive inhibitor of vinblastine efflux; vinblastine being itself a substrate for P-glycoprotein [23, 24]. However, it is difficult to equate BCECF efflux with P-glycoprotein function. BCECF efflux was not reduced by amiloride, nifedipine, reserpine or chloroquine, and was only moderately affected by verapamil and trifluoperazine, recognised inhibitors of P-glycoprotein [13, 14].

The rate of BCECF efflux is lower during the first hour as compared with subsequent efflux (Fig. 1, [4]). This might be explained by intracellular endosomal fluorochrome accumulation with subsequent translocation an exocytosis at the plasmalemma. Vinblastine, an effective inhibitor of BCECF efflux, disrupts microtubules and binds to tubulin so preventing polymerization into microtubules [25]. Nocodazole has a similar action and interferes with vesicular traffic in epithelial cells [15] but did not influence cumulative BCECF efflux at 5 hr. Other agents which interfere with vesicular trafficking, such as cytochalasin B and chloroquine, also did not reduce the cumulative BCECF efflux at 5 hr. These observations are thus consistent with previous reports which have suggested that intracellular organelle accumulation of BCECF is low [1, 26] and that BCECF efflux may be mediated by a transport system localized to the plasmalemma.

In conclusion, the pharmacological profile for inhibition of BCECF efflux does not allow us to equate it with any recognised transport system. BCECF might thus be representative of substrates for an additional class of ATP-dependent transport mechanisms. The physiological role which such a transport mechanism may subserve is likely to be as a cellular secretory detoxifying system, consistent with its demonstration in epithelial cells of both intestinal and renal origin [4]. Our recent demonstration of the polarized nature, apical efflux > basolateral efflux, of BCECF efflux in colonic (Caco-2) and renal (MDCK) epithelial cells [27] lends further support to such a proposed function.

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