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PROTON RELEASE FROM HeLa CELLS AND ALKALIZATION OF CYTOPLASM INDUCED BY DIFERRIC TRANSFERRIN OR FERRICYANIDE AND ITS INHIBITION BY THE DIARYLSULFONYLUREA ANTITUMOR DRUG *N*-(4-METHYLPHENYLSULFONYL)-*N'*-(4-CHOLOROPHENYL) UREA (LY181984)

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Abstract—Proton release from HeLa cells was stimulated by an external oxidant, potassium ferricyanide, or by the growth factor diferric transferrin. This stimulated proton release was inhibited by the antitumor sulfonylurea LY181984 [*N'*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea] over the concentration range 10 nM to 1 μM. The antitumor-inactive sulfonylurea analog LY181985 [*N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea] was without effect at 1 μM and required 10–100 μM concentrations to inhibit proton release. Diferric transferrin-induced alkalization of the cytoplasm estimated by BCECF [2',7'-bis(2-carboxyethyl)-5,(and 6)-carboxyfluorescein] fluorescence also was inhibited by 1 μM LY181984 but not by 1 μM LY181985. The inhibited component appeared to be amiloride resistant. The proton release induced by either ferricyanide or diferric transferrin was inhibited by about 35% at a near optimal amiloride concentration of 0.2 mM or at a dimethylamiloride concentration of 0.075 mM. However, the induced proton release was inhibited further by LY181984. Conversely, when proton release was inhibited fully by LY181984 at a near optimal concentration of 10 μM (50% inhibition), increasing concentrations of amiloride or dimethylamiloride resulted in additional inhibitions of 16 and 23%, respectively. However, the inhibitions by LY181984 and the amilorides were additive, suggesting that amiloride and the sulfonylureas may act independently. Evidence for an action of the sulfonylurea in inhibiting proton efflux differently from that of the amilorides came from measurements of sodium uptake either by fluorometry or by direct measurement with $^{22}Na^+$. Sodium uptake was not inhibited by either LY181984 or LY181985 in HeLa cells at concentrations of LY181984 sufficient to inhibit proton efflux by 80% or more. The results show LY181984 to be a potent inhibitor of diferric transferrin- or ferricyanide-induced proton efflux and cytoplasmic alkalization in HeLa cells and that the inhibition may involve a component of proton transport that is resistant to amiloride.

Key words: diarylsulfonylurea; sulfonylurea; antitumor drug; proton release; Na^+/H^+ antiport; amiloride; HeLa

The sulfonylureas represent a novel series of synthetic organic compounds identified as having activity against human solid tumors *in vivo* [1, 2]. They were identified as the result of a program of screening against *in vivo* murine solid tumors implanted subcutaneously [3, 4]. Known collectively as diarylsulfonylureas (sulfonylureas), their mechanism of action, while unknown, is apparently unrelated to previously described classes of oncolytic agents [2]. One member of the series, Sulofenur, progressed in evaluation to Phase I [2, 5] and Phase II [6] clinical trials.

Despite considerable clinical and laboratory data, the site of Sulofenur action has remained elusive [4, 7]. There is no evidence for cell cycle specificity of the drugs and no inhibition of DNA, RNA or protein syntheses [5, 7]. The sulfonylureas exhibit few, if any, mechanistic parallels to other known antitumor agents. The drugs are membrane active and weak uncouplers of mitochondrial oxidative phosphorylation [8–10]. Their mode of action is expected to be unique.

Our interest in the sulfonylureas stemmed initially from the demonstration of a requirement for quinones in the proton release from HeLa cells stimulated by differic transferrin or ferricyanide [11]. Analog inhibition was used with intact cells. The coenzyme Q analogs, DCIQ¶ or ETHOXQ, inhibited both ferricyanide- and diferric transferrin-stimulated proton release in HeLa cells, and this release could be reversed by the addition of coenzyme Q [11]. Since a relationship was established previously for sulfonylurea interactions with quinone sites [12, 13], we examined the response of both ferricyanide- and diferric transferrin-induced proton release of HeLa cells to the antitumor sulfonylurea LY181984.

Binding of diarylsulfonylureas to quinone sites was demonstrated initially with the sulfonylurea herbicides (chlorsulfuron, sulfometuron methyl, metsulfuron methyl). These herbicides were inhibitors of acetolactate syn-

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¶ Abbreviations: BCECF [2',7'-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein]; DCIQ, 2,3-dimethoxy-5-chloro-6-naphthylmercapto-1,4-benzoquinone; ETHOXQ, 2-methoxy-3-ethoxy-5-methyl-6-hexadecylmercapto-1,4-benzoquinone; $(Fe)_2TF$, diferric transferrin; LY181984, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl) urea; LY181985, *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea; and SBFI, sodium-binding benzofuran isophthalate.

thase, the entry enzyme into the branched amino acid biosynthesis pathway [12]. A clue to the identity of the quinone binding nature of the herbicide-specific site came from the discovery that the amino acid sequence of the acetolactate synthase sulfonylurea-binding site was similar to the sequence of pyruvate oxidase, which binds ubiquinones as cofactors [13]. These and other data [12] eventually led to the proposal that the sulfonylurea site of acetolactate synthase was an evolutionary vestige of a quinone binding site. Consistent with this proposal was evidence that ubiquinone homologs (Q_0 and Q_1) each competed with a radiolabeled sulfonylurea herbicide (sulfometuron methyl) for a common binding site on acetolactate synthetase.

As with the quinone analogs [11], both ferricyanide- and diferric transferrin-induced proton release were inhibited by low concentrations of the sulfonylurea as was diferric transferrin-induced alkalization of the cytoplasm. Proton efflux activated by mitogenic growth factors and consequent alkalization of the cytoplasm has been related to growth control mechanisms [14–17]. Proton release from HeLa cells can be stimulated by a number of growth factors including diferric transferrin [14], as well as by external oxidants such as ferricyanide [18]. The latter stimulates growth of HeLa cells in serum-deficient media [19, 20]. Comparisons with amiloride and measurements of effects on sodium uptake suggest, however, that inhibition of proton efflux and cytoplasmic alkalization in LY181984-treated cells may result from inhibition at a site distinct from that of the Na^+/H^+ antiport targeted by amiloride. The sulfonylurea inhibition is reduced or prevented by added ubiquinone, but more definitive information to relate sulfonylurea and quinone binding must await molecular characterization of the protein(s) involved.

MATERIALS AND METHODS

Growth of cells

HeLa cells (ATCC CCL2) were grown in 150 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37° with 10% bovine calf serum (heat-inactivated), plus 50 mg/L gentimycin sulfate (Sigma). Cells were trypsinized with Sigma IX trypsin for 1–2 min, harvested by scraping, and taken up in TD-Tris buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4) to a final cell concentration of 0.1 g wet weight/mL.

HeLa S cells were grown in Minimal Essential Medium (S-MEM) (Jolik modified) with glutamine (244 mg/L) and phosphate (1.3 g/L Na_2HPO_4) and without $CaCl_2$ plus 5% donor horse serum. Gentimycin sulfate (50 mg/L) and sodium bicarbonate (2 g/L) were added. Cells were collected by centrifugation for 6–15 min at 150–1000 *g* (e.g. 6 min at 1000 *g* or 15 min at 150 *g*). Cell survival was determined by Eosin Y exclusion.

Proton release

Proton release was measured in a water-jacketed 3-mL cuvette with an Orion 701-A pH meter and a Corning combination glass electrode at a final cell concentration of 0.2 g wet weight/mL. The assay medium consisted of 0.1 M sucrose, 10 mM KCl, 10 mM NaCl, and 10 mM $CaCl_2$ bubbled continuously to remove CO_2. The system was equilibrated for 5 min at 37°, and the reaction was started with the addition of diferric transferrin (10 μM) or potassium ferricyanide (0.1 mM). Cells were incubated with drugs for 3 min before assay. After 5 min at 37°, 5 μL of 0.01 M HCl (= 50 nmol H^+) was added as an internal standard.

Determination of cytoplasmic pH

Change in the cytoplasmic pH was monitored by the method of Rink *et al.* [21]. The cell suspensions (10^7 cells/mL) were loaded with BCECF by incubation with a 5 μM concentration of the parent acetoxymethyl ester, BCECF-AM (Sigma), for 30 min at 37° in the dark. The cells were then collected by centrifugation and washed once with Earle's balanced salt solution, pH 7.2, to remove untrapped dye, and resuspended in the same medium at a final concentration of 10^7 cells/mL. Fluorescence was measured using a Coulter (Elite) flow cytometer with a 15 mV argon laser operating at a wavelength of 488 nm for excitation. Fluorescence intensity was monitored with two separate 20 nm wavelength bands centered at 525 and 635 nm, respectively, and expressed as the ratio of emission intensities. Intracellular calibration of the BCECF fluorescence response was with cells loaded with the dye in parallel and resuspended in high $[K^+]$ buffers containing the proton ionophore nigericin at a final concentration of 1 μM [22].

Sodium uptake

For measurement of sodium uptake [23], cells were grown in 25 mm dishes and equilibrated at 37° for 15 min in Earle's salt solution (150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM HEPES-Tris, pH 7.4). This medium was replaced by a modified Earle's salt solution containing 3 mM NaCl supplemented with 0.2 mM ouabain and 1 μCi/mL of $^{22}Na^+$ (500 mCi/mg, Amersham) with and without sulfonylurea or with or without dimethylamiloride. The cells were incubated for 1 min at 37°, washed rapidly with 0.1 M $MgCl_2$, and finally dissolved in 0.1 N sodium hydroxide. Radioactivity was determined using a Packard model 3255 scintillation spectrometer.

To estimate cytosolic Na^+ by a fluorescence method, the sodium indicator SBFI [24] was utilized. Two solutions of equal ionic strength were employed. One was Na^+ free and contained 130 mM potassium gluconate and 30 mM KCl. The other contained 130 mM sodium gluconate and 30 mM NaCl. Both solutions contained 10 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgSO_4$ and were titrated with *N*-methyl-D-glucosamine base to pH 7.1 [25]. Spectra were obtained with an SLM 8000.

Amiloride was from Sigma. Dimethylamiloride was from Merck, Sharp & Dohme. The sulfonylureas were dissolved in DMSO at a concentration of 10 mM and diluted with DMSO. Control cells received an equivalent amount (3 μL/3 mL) of DMSO.

RESULTS

Proton release by HeLa cells stimulated by diferric transferrin was inhibited 50% by the active sulfonylurea antitumor agent LY181984 at a concentration of 0.5 μM (Fig. 1). In contrast, LY181985, a relatively inactive sulfonylurea in inhibition of growth ([4], Table 1), was without effect at 1 μM on proton release (Fig. 1). Even at 100 μM, proton release was inhibited less than 50% (Fig. 1).

In conducting these experiments, cells were first

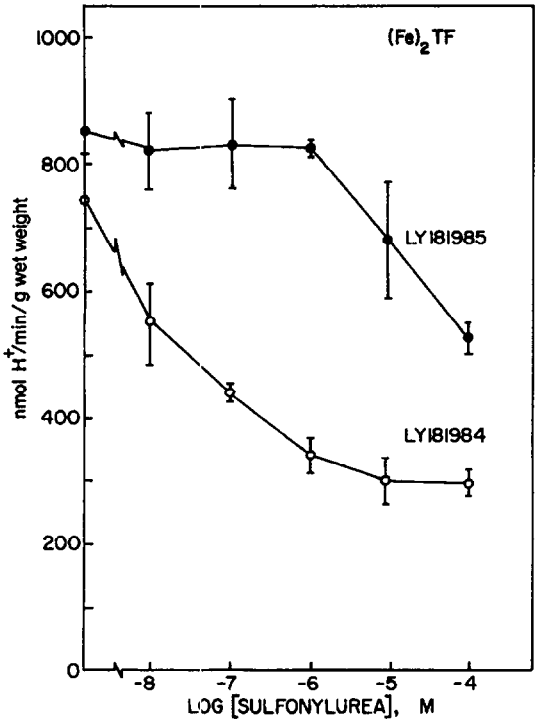

Fig. 1. Inhibition of differic transferrin [$(Fe)_2TF$, 10 μM] induced proton efflux from HeLa cells by LY181984, a sulfonylurea active in growth inhibition, but not by the chemically closely related LY181985, a sulfonylurea inactive in growth inhibition. Once initial equilibration was achieved (within 100 sec or less), none of the compounds at the concentrations tested affected pH in the absence of cells. Results are means ± SD, N = 3.

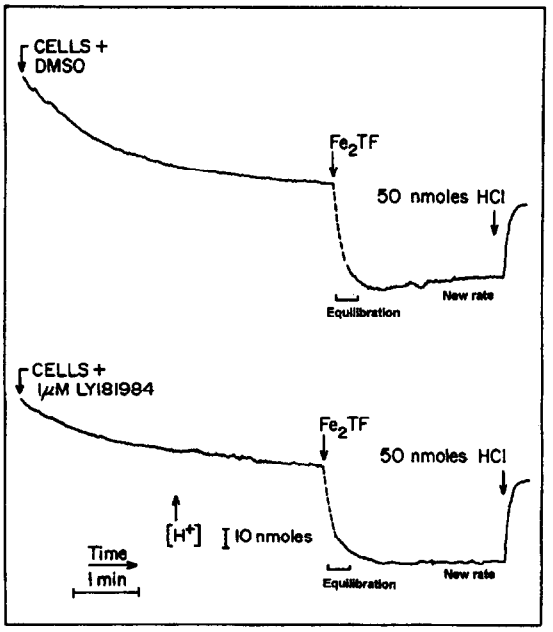

Fig. 2. Induction of proton release from HeLa cells by $(Fe)_2TF$. Cells were equilibrated in the indicated buffer solution until a steady-state rate of proton exchange was obtained. Then $(Fe)_2TF$ (10 μM) was added at the second arrow to induce proton release. The discontinuity during the first 60 sec following $(Fe)_2TF$ addition, shown by the dotted line, results from a dilution response to the $(Fe)_2TF$ addition and is not a measure of proton release. The $(Fe)_2TF$-induced rate of proton release was established within 1 min and was unaffected by DMSO (upper trace) or 1 μM LY181985 added in DMSO (not shown) but was reduced markedly by 1 μM LY181984 added in DMSO (lower trace). The final concentration of DMSO was 0.01%. The pH change was measured with a combination glass electrode. All solutions contained 1.5 mM Tris chloride to dampen pH change. Proton release was calibrated by adding 50 nmol HCl at the end of each incubation. The chamber was bubbled constantly with air to remove CO_2. Neither LY181984 nor LY181985 alone had an effect on pH in the absence of cells.

equilibrated in the buffer solution until proton release/exchange was at a steady state (Fig. 2). At the points indicated, diferric transferrin was added to induce proton release. At the end of each determination, the proton release was calibrated by the addition of 50 nmol HCl The stimulation of proton efflux induced by diferric transferrin was considerable as was the degree of inhibition by LY181984 (Figs. 1 and 2).

With ferricyanide-stimulated proton release, results were similar to those with diferric transferrin-stimulated proton release (Fig. 3). The active LY181984 inhibited ferricyanide-stimulated proton release by 43% at 1 μM and by 60% at 10 μM (Fig. 3).

Also inhibited by 1μM LY181984 (active) but not by LY181985 (inactive) was diferric transferrin-induced alkalization of the cytoplasm as determined by BCECF fluorescence (Fig. 4). A steady-state rise in pH, determined from the increase in the ratio of fluorescence emission intensities at 515–525 nm/615–635 nm, of *ca.* 0.005 pH units/min was observed following the addition of 10 μM diferric transferrin (Fig. 4). This rate of increase was unaffected by addition of DMSO alone or LY181985 (inactive) in DMSO but was inhibited nearly completely by 1 μM LY181984 (active) (Table 2, Fig. 4). Addition of LY181984 to the differic transferrin-treated cells resulted in a rather abrupt cessation of the differic transferrin-induced rise in pH followed at about 400 sec by a decrease in cytoplasm pH.

Absolute pH change was estimated from calibration curves constructed using cells loaded with BCECF, incubated in parallel with the experimental sample, in which the cells were resuspended in high $[K^+]$ buffers containing the protonophore nigericin. The effect of LY181984 on the inhibition of cytoplasmic alkalization occurred within 100 sec after sulfonylurea addition and,

Table 1. Increase in cell number (growth) of HeLa cells over 72 hr of continuous culture in the presence of various concentrations of the antitumor sulfonylurea LY181984, its inactive analog LY181985, amiloride and dimethylamiloride

Addition	Concentration	Cells/mm²
None		250 ± 12
Amiloride	0.1 mM	172 ± 27
	0.3 mM	146 ± 50
Dimethylamiloride	0.1 mM	114 ± 73
	0.3 mM	24 ± 15
DMSO	0.1%	220 ± 20
LY181984 in DMSO	100 μM	60 ± 18
LY181985 in DMSO	100 μM	200 ± 15

Initial plating density was 100 ± 12 cells/mm². Values are means ± SD, N = 3.

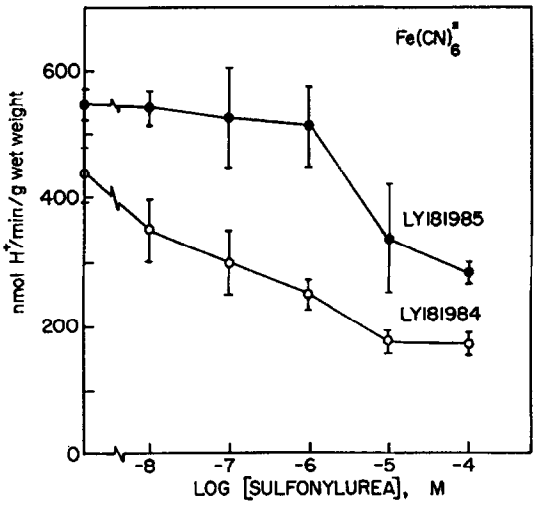

Fig. 3. Inhibition of ferricyanide- (0.1 mM) induced proton efflux from HeLa cells by LY181984 and LY181985. Once initial equilibration was achieved (within 100 sec or less), none of the compounds at the concentrations tested affected pH in the absence of cells. Results are means ± SD, N = 3.

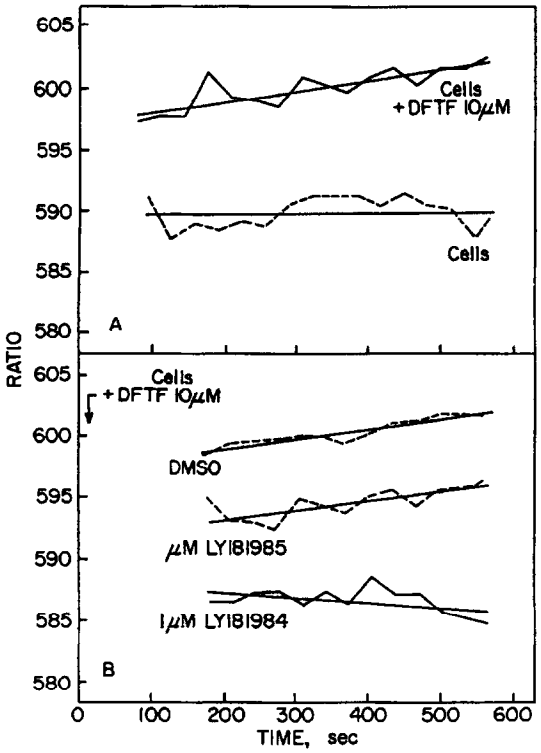

Fig. 4. Changes in fluorescence emission measured as the ratio of 525/635 nm as a function of time for attached HeLa cells where the ratio is proportional to pH (0.1 pH unit = 12 ± 3 units of ratio change). (A) Cytoplasmic alkalization corresponding to transferrin compared with cells alone. (B) Effect of addition of 1 μM LY181984 to the diferric transferrin (DFTF)-treated cells compared with the addition of the inactive LY181985 or DMSO alone. The regression line shown for LY181984 was significantly different from that for LY181985 or DMSO ($P < 0.001$).

in the experiment of Fig. 4, had achieved a slope of −0.0225 ($r = 0.97$) between 400 and 600 sec of sulfonylurea addition.

With HeLa S cells where the cytoplasmic alkalization appears to be activated constitutively (independent of diferric transferrin addition), LY181984 at 1 μM but not LY181985 inhibited steady-state alkalization as well (Table 2). These results were repeated several times with consistent findings.

In a separate series of experiments using BCECF fluorescence, cells grown at pH 7.2 were incubated in buffer at pH 5.5, and the intracellular pH was monitored. In untreated cells or in cells treated with 100 μM sulfonylurea or less, no significant decrease in internal pH was noted over 10 min of incubation. However, at LY181984 concentrations of 100 μM or greater, pH control was compromised but not to the same extent as was observed with 1 μM nigericin.

Both the diferric transferrin- and the ferricyanide-induced rates of proton efflux were augmented but not dependent on the presence of sodium in the medium (Table 3). The ferricyanide- or differic transferrin-induced proton efflux was supported by choline chloride although less effectively than by sodium chloride.

Amiloride, an inhibitor of the sodium-dependent proton antiport, partially inhibited proton release induced by both transferrin and ferricyanide (Fig. 5). The inhibition was maximally inhibited (about 35%) at 0.2 mM amiloride.

When induced proton release was inhibited by 0.2 mM amiloride (Fig. 6), the addition of sulfonylurea resulted in a further concentration-dependent inhibition. With the active LY181984, inhibitions of 50 and 85% were achieved with the two drugs in series at 10 μM sulfonylurea comparing diferric transferrin- (Fig. 6A) and ferricyanide- (Fig. 6B) induced proton release, respectively. With the inactive LY181985, inhibitions of 40 and 80% were obtained at 100 μM sulfonylurea. As with inhibition of proton efflux in the absence of amiloride, the concentration of LY181985 was two log orders of magnitude higher than that of LY181984 to achieve the same effect (Fig. 6).

Likewise, when the proton efflux was inhibited maximally by 10 μM LY181984, amiloride exerted an additional inhibitory effect to a near optimum at 0.2 mM (Fig. 7). Maximum total inhibitions of about 65 and 70% were achieved by the combination of 10 μM LY181984 plus 0.2 mM amiloride for diferric transferrin- (Fig. 7A) and ferricyanide- (Fig. 7B) induced proton release, respectively.

Dimethylamiloride, an amiloride derivative capable of blocking amiloride-resistant proton efflux (Fig. 8), appeared to block a component of the sulfonylurea-inhibited proton efflux, but an additive response was still obtained. Diferric transferrin-induced proton release was inhibited by about 45% at 0.1 mM. The combination of 0.075 mM dimethylamiloride followed by 10 μM LY181984 increased the inhibition to 80% (Fig. 9A). With 10 μM LY181984 followed by dimethylamiloride, inhibitions of proton efflux of 60% were obtained (Fig. 9B).

LY181984 (active) but not LY181985 (inactive) inhibited uptake of $^{22}Na^+$ but only at much higher concentrations than were required to inhibit proton efflux (Fig. 10). In some experiments (not shown), LY181984 appeared to stimulate uptake of $^{22}Na^+$ over the concentration range 10 nM to 1 μM.

Table 2. Relative rate of change of intracellular pH of HeLa and HeLa S cells to diferric transferrin and response to two sulfonylureas as determined from the ratio of BCECF fluorescence emission at 525/635 nm

Cell line	Diferric transferrin	Sulfonylurea	Relative slope	Correlation coefficient
HeLA (attached)	None	None	0.0009	0.11
	10 μM	None	0.0103	0.87
		DMSO	0.0116	0.97
		1 μM LY181984	−0.0037	−0.46
		1 μM LY181985	0.0078	0.76
HeLa S (suspension)	None	None	0.0201	0.87
	10 μM	None	0.0189	0.84
		DMSO	0.0236	0.75
		1 μM LY181984	0.0144	0.70
		1 μM LY181985	0.0292	0.93

Cells were suspended in 5 μM BCECF-AM for 30 min at 37° after which diferric transferrin (10 μM) was added in the presence of 1 μM active (LY181984) or inactive (LY181985) antitumor sulfonylurea. Cytoplasmic alkalization was determined from the increase in the ratio of BCECF fluorescence monitored continuously over 10 min following addition of growth factor or drug. Results are given as computer-derived slopes between 150 and 600 sec. The first 150 sec was eliminated from the analysis as the time for the system to equilibrate and to achieve a new steady-state rate. The diferric transferrin-induced rate of cytoplasmic alkalization and the constitutive rate with HeLa S cells grown in suspension were very similar and approximately equal to 0.005 pH units/min, as determined by calibration with standard buffers of known pH in the presence of 1 μM nigericin.

Sodium uptake also was measured using the sodium indicator SBFI (Fig. 11). Within 1–3 min after transfer of cells from sodium-free medium to medium containing 150 mM sodium, the spectral response of SBFI to cells preloaded with SBFI for 30 min was indistinguishable in the presence of 1 μM LY181984, 1 μM LY181985 or with DMSO alone.

DISCUSSION

The findings reported here suggest that the proton efflux induced by both diferric transferrin [14], a growth factor, and ferricyanide [18], an external oxidant capable of promoting growth in serum-deficient media [19, 20], appears to be sensitive to inhibition by an antitumor sulfonylurea. Proton efflux and the consequent alkalization of the cytoplasm have been related to mechanisms of growth control [14–17]. While the proton efflux inhibited by the anticancer sulfonylurea LY181984 is distinct from that of the Na^+/H^+ antiport, we have not answered the question of whether or not the inhibition of proton efflux by sulfonylureas is related to inhibition of growth in cell culture. The concentrations of LY181984 observed to inhibit proton efflux were more nearly in the concentration ranges where sulfonylureas inhibit growth of tumors *in vivo* [4]. They were lower than those re-

Table 3. Effect of sodium ions on the rate of net proton release by HeLa cells (2-day culture)

Effector	Addition	H^+ release (nmol/min/g wet weight)
0.1 mM Ferricyanide	250 mM Sucrose	50 ± 25
	150 mM Choline chloride	151 ± 8
	10 mM NaCl	790 ± 67
	150 mM NaCl	406 ± 31
10 μM Diferric transferrin	250 mM Sucrose	100 ± 50
	150 mM Choline chloride	195 ± 28
	10 mM NaCl	525 ± 65
	150 mM NaCl	499 ± 26

Values are means ± SD, N = 3.

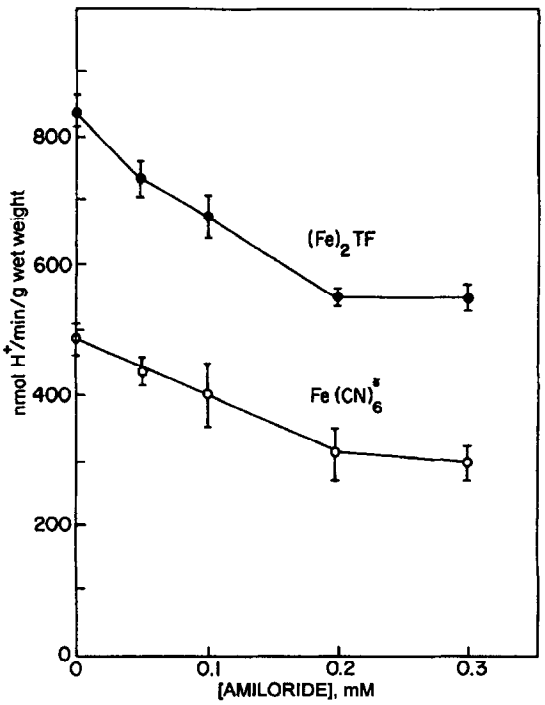

Fig. 5. Amiloride inhibition of diferric transferrin- (10 μM) and ferricyanide- (0.1 mM) induced proton efflux. Results are means ± SD, N = 3.

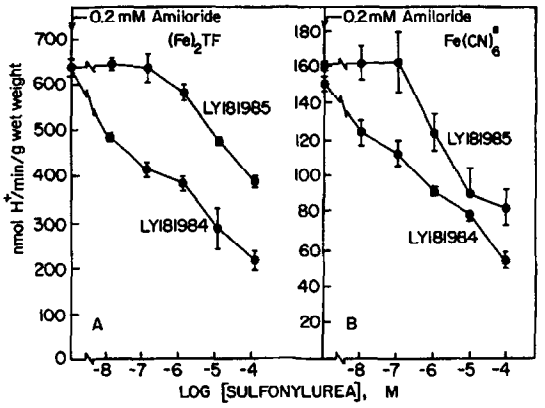

Fig. 6. Inhibition of proton efflux by 0.2 mM amiloride followed by increasing concentrations of sulfonylurea. The ordinate values are specific proton efflux after equilibration with 0.2 mM amiloride. (A) Diferric transferrin- (10 μM) induced proton efflux. (B) Ferricyanide- (0.1 mM) induced proton efflux. Results are means ± SD, N = 3.

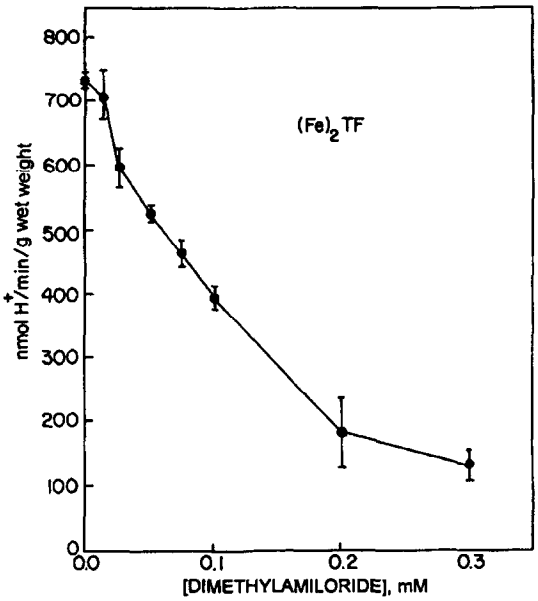

Fig. 8. Dimethylamiloride inhibition of diferric transferrin (10 μM) induced proton efflux. Results are means ± SD, N = 3.

quired to inhibit growth of HeLa cells in culture under the growth conditions used in this study [26].

The proton release by HeLa cells is a mixture of amiloride-sensitive (antiport) and -insensitive (sulfonylurea target) proton efflux mechanisms. Both Sun *et al.* [18] Garcia-Cañero and Guerra [27] have shown previously that proton release by HeLa cells in response to ferricyanide is stimulated by sodium ions and is, at least partly, amiloride sensitive. Additionally, Garcia-Cañero and Guerra [27] have shown that a component of sodium uptake is stimulated by ferricyanide. However, perhaps no more than 50% of the proton efflux by HeLa cells was amiloride sensitive (Fig. 5). It was the amiloride-insensitive component of proton efflux that was sensitive to sulfonylurea. Even with dimethylamiloride, which inhibits an amiloride-resistant component of proton efflux, the inhibitions by dimethylamiloride and active antitumor sulfonylurea were additive. Also, we were unable to demonstrate an inhibition of sodium uptake by the sulfonylureas comparable to the inhibition of induced proton efflux. The latter also pointed to inhibition by sulfonylureas of plasma membrane-located proton efflux by a unique mechanism.

LY181984 inhibited proton efflux by HeLa cells in a log concentration-dependent manner with significant reduction in proton efflux at concentrations as low as 10 nM. The inactive LY181985 was not without effect, but 2–3 log orders higher concentrations of drug were required to achieve the same effects. Thus, inhibition of proton efflux is not an unspecific response to the sulfonylureas in general but may bear some relations to their cytotoxic action. A more detailed concentration response for growth of HeLa cells in response to increasing concentrations of LY181984 is given by Morré and Morré [26].

Although the active (LY181984) but not the inactive (LY181985) sulfonylurea inhibited $^{22}Na^+$ influx, sub-

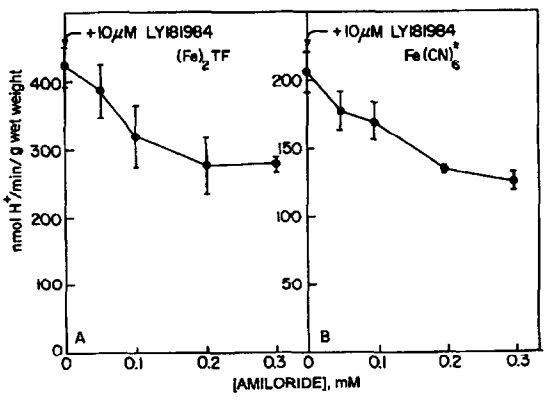

Fig. 7. Inhibition of proton efflux by 10 μM LY181984 followed by increasing concentrations of amiloride. The ordinate values are specific proton efflux after equilibration with 10 μM LY181984. (A) Diferric transferrin- (10 μM) induced proton efflux. (B) Ferricyanide- (0.1 mM) induced proton efflux. Results are means ± SD, N = 3.

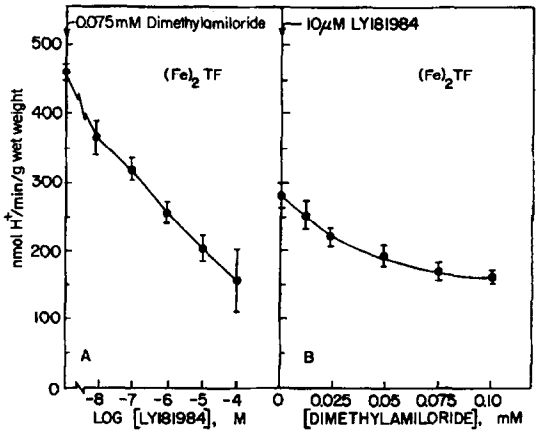

Fig. 9. Inhibition of diferric transferrin-induced proton efflux by dimethylamiloride followed by increasing concentrations of LY181984 (A) or by LY181984 followed by increasing concentrations of dimethylamiloride (B). Results are means ± SD, N = 3.

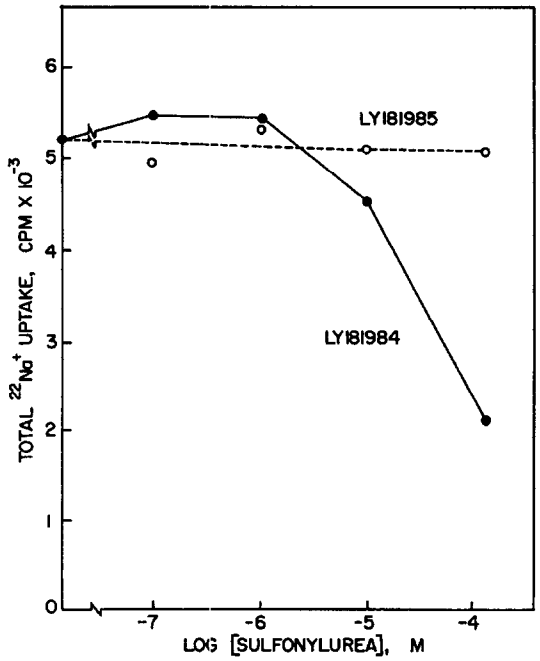

Fig. 10. $^{22}Na^+$ uptake by HeLa cells with increasing concentrations of LY181984 (active) and LY181985 (inactive).

stantially greater concentrations were required than to inhibit proton efflux. In parallel experiments, $^{22}Na^+$ uptake was measured as described by Garcia-Cañero *et al.* [28] and Diaz-Gil *et al.* [29]. Again, the sulfonylureas at 1 μM final concentrations were ineffective in inhibiting either the basal $^{22}Na^+$ uptake or $^{22}Na^+$ uptake stimulated by ferricyanide or diferric transferrin. These results point to inhibition of some mechanism involving proton efflux other than the inhibition of an inward Na^+ electrochemical gradient.

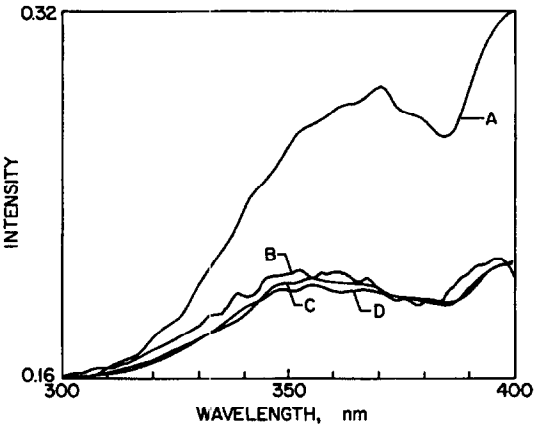

Fig. 11. Excitation spectra of the sodium indicator SBFI (sodium-binding benzofuran isophthalate) [23] comparing HeLa cells loaded in the presence of 5 μM SBFI-AM under different experimental conditions. (Curve A) Transfer to sodium-free medium. (Curve B) After transfer to sodium-containing medium with DMSO alone. (Curve C) After transfer to sodium-containing medium with 1 μM LY181984 (active). (Curve D) After transfer to sodium-containing medium with 1 μM LY181985 (inactive). Spectra were obtained within 1–2 min after transfer to sodium-containing media.

In parallel to inhibition of proton efflux by micromolar concentrations of LY181984, we observed, as well, a concomitant diminution of cytoplasmic alkalization as determined from BCECF fluorescence. In attached HeLa cells, a marked diferric transferrin-induced component of cytoplasmic alkalization was observed. This induced component was inhibited almost completely by 1 μM LY181984. With HeLa S cells, where constitutive cytoplasmic alkalization was observed, 1 μM LY181984 but not 1 μM LY181985 again inhibited this induced component. The absence of a response to decreasing pH of the LY181984 inhibition of $(Fe)_2$TF-induced proton efflux would imply that short-term sulfonylurea effects were independent of sulfonylurea uptake, as the latter has been shown to be pH dependent [30].

Fine regulation of intracellular pH (pH_i) is normally thought to be accomplished via active extrusion of H^+ by a Na^+/H^+ antiport localized at the plasma membrane and driven by the energy of the inward Na^+ electrochemical gradient [14]. The set point for pH_i is typically *ca.* 7.2 for resting cells but stimuli, including most if not all hormones and growth factors, as well as tumor-promoting phorbol esters, apparently shift the set point to a pH_i of 7.4 to 7.5 [14–17]. It is the latter that is apparently blocked by the antitumor sulfonylurea. The energy source for the sulfonylurea-blocked proton efflux is not known but may not be Na^+ influx since the diuretic amiloride, which binds to the external Na^+ site with an inhibition constant of 7–30 mM [31], did not specifically compete with the antitumor sulfonylurea in the inhibition of proton efflux.

Inhibition of proton efflux with a concomitant lowering of the pH_i set point could affect the growth of tumor cells *in situ* even more profoundly than in cell culture. In culture, cells are surrounded by abundant growth factors and a physiological pH of 7.2. However, *in situ*, tumors normally encounter a more acidic environment and potentially a paucity of growth factors. Furthermore, tumor cells may place more reliance on a sulfonylurea-inhibited proton efflux mechanism and less on the classic Na^+/H^+ antiport than do normal cells. Thus, the sulfonylurea-inhibited proton efflux mechanism may represent a novel drug target of relevance to growth control in cancer as well as to the oncolytic response of cancer cells to the antitumor sulfonylureas.

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