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Characterization of Na^+ Transport in Normal Human Fibroblasts and Neoplastic H.Ep.2 Cells and the Role of Inhibitin

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Summary. Na^+ transport was characterized in normal human fibroblasts and neoplastic H.Ep.2 cells in order to investigate the role of the endogenous peptidic factor 'inhibitin' that is secreted by a variety of neoplastic cells (including H.Ep.2) and inhibits Na^+/Na^+ exchange in human erythrocytes. Although active (Na^+,K^+-ATPase mediated) Na^+ fluxes were similar in the two cell types, H.Ep.2 cells maintained higher intracellular Na^+ concentration (26 mM) compared to fibroblasts (12 mM). An analysis of passive Na^+ fluxes showed a difference in the handling of Na^+ via ouabain and bumetanide-insensitive transport between the two cell types: H.Ep.2 cells achieved net Na^+ influx via an amiloride-sensitive pathway that was only demonstrated in fibroblasts when 10% fetal calf serum (FCS) was present. Kinetic studies were undertaken to investigate the interaction between Na^+ flux via Na^+/H^+ and Na^+/Na^+ exchanges. For this purpose, an outwardly directed Na^+ gradient was created by loading the cells with Na^+ ($Na_i > 100$ mM) to activate the reverse functioning of Na^+/H^+ exchange (i.e., Na^+_{out} H^+_{in}). The rates of ouabain- and bumetanide-insensitive Na^+ efflux were measured over a range of extracellular Na^+ concentrations (Na^+_o 14–140 mM). In the presence of 10% FCS, the two cell types showed different responses: in fibroblasts the Na^+ efflux rate showed an inverse correlation with extracellular Na^+ concentration, while H.Ep.2 cells significantly increased their rate of Na^+ efflux as extracellular Na^+ concentration increased. So although the thermodynamic force would direct net Na^+ efflux when $Na^+_i > Na^+_o$, H.Ep.2 cells were under kinetic control to perform Na^+/Na^+ exchange.

When exogenous inhibitin was tested on fibroblasts, the steady-state intracellular Na^+ concentration increased from 14 to 19 mM ($p < 0.01$). In Na^+-loaded fibroblasts, serum-stimulated Na^+ efflux was partially inhibitin sensitive and the maximal inhibitory effect was seen when extracellular Na^+ concentration was 14 mM and presumably the Na^+/H^+ exchanger operating in the reverse mode. This study demonstrated that, in contrast to fibroblasts, H.Ep.2 cells have a modified Na^+/H^+ exchange system whereby it acts in the Na^+_{in} H^+_{out} mode without exogenous growth factor activation and resists functioning in the reversed mode. It is proposed that inhibitin is the endogenous modifier of this transport system in H.Ep.2 cells with the result that H.Ep.2 cells maintain a higher concentration of intracellular Na^+ compared to fibroblasts.

Key Words sodium transport · fibroblasts · H.Ep.2 cells

Introduction

The animal cell is in osmotic but not electrochemical equilibrium with its surroundings. An unequal distribution of ions causes the cell to be electronegative with respect to the plasma, and this is achieved by the outward transport of Na^+ (the principal plasma cation) in exchange for K^+ (the principal intracellular cation) mediated by the enzyme, Na^+, K^+-ATPase commonly known as the Na^+ pump [20, 47]. Other forms of transmembrane Na^+ and K^+ movement are collectively termed as 'passive' transport and include: exchange diffusion which is the bidirectional flux of Na^+ and K^+ without any net transport [17, 30, 31], Na^+, K^+ cotransport is the coupled Na^+ and K^+ movement sensitive to the loop-diuretics [2, 19, 32, 49, 65], Na^+/H^+ countertransport couples the 'downhill' influx of Na^+ to the 'uphill' extrusion of H^+ [1, 43], and there is evidence to show that this represents an alternative mode to Na^+/Na^+ exchange [3, 6, 25]. Electrodiffusional leak is the passage of Na^+ and K^+ down their respective concentration gradients and is dependent on the ion permeability of the cell membrane [20].

The regulation of intracellular Na^+ and K^+ concentrations plays many important roles. The potential energy stored by the Na^+ gradient is utilized to drive other transport systems such as glucose and amino acid uptake [16, 62], and Na^+, K^+ cotransport is thought to contribute to the regulation of cell volume [19]. Changes in monovalent ion fluxes are important events following growth factor activation of quiescent cells in culture [7, 52, 54, 56]. Na^+/H^+ exchange is the principal pathway that plays a pivotal role [35, 36, 37, 48] in causing an increase in Na^+ influx, which in turn stimulates the Na^+ pump [33, 57, 59], thereby increasing intracellular K^+ concentration required for protein synthesis during the

replicative phase [28, 29]. In addition, a rise in H^+ efflux causes intracellular alkalinization [13, 53], which can cause major changes in enzyme activity [10, 23].

There is evidence to suggest that these changes in ion transport are of fundamental importance in neoplastic cells; alterations in membrane Na^+ permeability and increased Na^+ pump activity have been observed in the transformed phenotype [11, 22, 24, 55]. A variety of cultured malignant cells have been found to secrete a peptidic factor (inhibitin) that specifically inhibits Na^+/Na^+ exchange in red blood cells [40–42]. These studies have suggested the intriguing possibility that this factor must play a role in the Na^+ handling of the secreting cells. This study was designed to explore this question and to characterize the Na^+ transport pathways in normal human fibroblasts and in an inhibitin-secreting neoplastic cell line, H.Ep.2 cells [41], to investigate whether any intrinsic differences in Na^+ handling could be attributable to the endogenous production of inhibitin. Purified inhibitin was also tested on Na^+ transport of fibroblasts. These studies were carried out in the absence and presence of serum to monitor the effect of growth factor activation of Na^+ transport and to ascertain the possible role of inhibitin under these conditions.

Materials and Methods

Growth media and plastic ware for cell cultures were obtained from Flow Laboratories (Irvine, Scotland). ^{22}Na was purchased from Amersham International PLC (Amersham, Buckinghamshire). Ouabain, amiloride and spectrosol trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (Poole, Dorset) and bumetanide was from Leo Laboratories (Princes Risborough, Buckinghamshire). Sep-Pak C_{18} cartridges were purchased from Waters Associates (Milford, MA) and the Hypersil ODS 5 μm reverse-phase high performance liquid chromatography (HPLC) column from Jones Chromatography (Llanbradach, Mid Glamorgan). HPLC grade S acetonitrile (MeCN) was from Rathburn Chemicals (Walkerburn, Scotland), HPLC/spectrograde heptofluorobutyric acid (HFBA) was purchased from Pierce UK Ltd (Cambridgeshire). All other chemicals used were Analar Grade from BDH Chemicals (Poole, Dorset).

Cell Cultures

1) Human fibroblasts: These cells were grown from small sections of human foreskin as previously described [59]. For sodium transport studies the cells were seeded in plastic petri dishes (50 mm diameter) at a density of 1×10^5 cells/ml (3 ml total volume). After 8 days with one change of medium, the fibroblasts were confluent and used for transport experiments.
2) H.Ep.2 cells (human carcinoma of the larynx): A stock culture of H.Ep.2 cells were originally purchased from Flow Laboratories (Irvine, Scotland). The cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin and grown at 37°C in an atmosphere of 5% CO_2 in air. For sodium transport studies the cells were trypsinized (0.25% wt/vol trypsin) and seeded into plastic petri-dishes (50 mm) at a density of 1×10^4 cells/ml (3 ml total volume). After 4 days, the H.Ep.2 cells had formed a confluent monolayer and were used for transport experiments.

A limited number of experiments was carried out on the following cell lines:

3) WI-38 (human embryonic lung). These cells have a finite life span and fibroblast morphology.
4) WI-38 VA13. These are WI-38 cells that have been transformed by SV-40 virus resulting in an epithelial morphology and continuous life span.
5) MDCK (dog kidney). These cells have an epithelial morphology and grow continuously.
6) HT1080 (human fibrosarcoma). These cells have an epithelial morphology and grow continuously.
7) LI32 (human lung). These cells have an epithelial morphology and grow continuously.
8) T4TD (human breast). These cells have an epithelial morphology and grow continuously.
9) Hypothalamic cells (fetal rat hypothalamus). These cells have fibroblast morphology and have a finite life span.

Cell lines 3–6 were originally purchased from Flow Laboratories, Irvine, Scotland. Cell lines 7 and 8 were kindly supplied by Dr. N. Thomas, Biomedical Division, Amersham International PLC, Whitchurch, Cardiff, and the hypothalamic cell line (9) was a gift from Mr. J. Kendall, Department of Medicine, University of Wales College of Medicine, Cardiff. All these cell lines were maintained in RPMI-1640, supplemented as described for H.Ep.2 cells. The preparation of the cells for transport experiments was as described for fibroblasts and H.Ep.2 cells.

Transport Studies

All sodium transport studies on cultured cells were carried out in medium based on Hank's balanced salt solution (HBSS (in mM): NaCl 137, $CaCl_2$ 1.26, $MgSO_4$ 0.4, $MgCl_2$ 0.49, Na_2HPO_4 0.17, KH_2PO_4 0.44, glucose 5.5, KCl 5.4, HEPES 20, pH 7.4). The various modifications of this physiological medium are described below.

Sodium Efflux

This method has been previously described [58, 59]. The culture medium was removed and the cells were washed three times with 5 ml of HBSS + ChCl (91 mM choline chloride had been substituted for 91 mM NaCl). The dishes of cells were then incubated in 2 ml of HBSS + ChCl + 1 μCi ^{22}Na. The purpose of substituting choline for sodium was to increase the specific activity of the loading medium. Preliminary studies had shown that with HBBS it was difficult to incorporate sufficient ^{22}Na intracellularly for accurate efflux measurements but by adopting this substituted medium, enough counts were incorporated without raising the intracellular sodium concentration.

After 2 hr incubation at 37°C, the radioactive medium was removed and the cells were washed and incubated in 3 ml of efflux medium (the composition of which depended on the transport process being measured and is outlined under the appropriate heading in Results). After various time intervals up to 10 min,

the dishes of cells were rapidly washed with 3 × 5 ml of ice-cold choline chloride solution (ChCl 151 mM, $MgCl_2$ 1 mM, pH 7.4) and then the cells were solubilized in 2 ml of 0.4 M NaOH, counted for radioactivity and subsequently estimated for protein content [27]. For each time point and for each set of efflux conditions, quadruplicate sets of petri dish cultures were used.

The first-order rate constant (the fraction of intracellular isotope lost from the cells per unit of time) was derived from log cpm/mg protein *vs.* efflux time in min. The sodium efflux rate has been shown to remain linear during the period of study [58]. The slope was calculated by the method of least squares, and the half-time ($t_{0.5}$) was derived from this. Sodium efflux rate constant ($^{\circ}K_{Na^+}$) was calculated from the equation:

$$^{\circ}K_{Na^+} = \frac{0.693}{t_{0.5}}$$

The 'total' sodium efflux rate constant $^{\circ}K^{T}_{Na^+}$ (*a*) was obtained when efflux was measured in the absence of ouabain. The 'passive' or ouabain-insensitive rate constant $^{\circ}K^{OI}_{Na^+}$ (*b*) was calculated from the efflux measured in the presence of 0.5 mM ouabain and the 'active' or ouabain-sensitive sodium efflux rate constant $^{\circ}K^{OS}_{Na^+}$ (*c*) was calculated by subtracting *b* from *a*. To express sodium efflux as μmol sodium/g protein/min, the following equation was used:

$$\text{Efflux} = {}^{\circ}K_{Na^+} \cdot (Na^+_i)$$

and (Na^+_i) was the internal sodium content obtained from

$$(Na^+_i) = \frac{\text{cpm/mg protein at } t = 0}{SA_{ex}}$$

and SA_{ex} was the specific activity of the extracellular medium (cpm/μmol sodium).

$^{22}Na^+$ *Influx*

For sodium influx measurement the cells were preincubated for 2 hr in HBSS + ChCl. This ensured that the cells were in the same physiological state as those used for efflux measurements. 2 ml of HBSS containing 1 mM ouabain (to prevent sodium efflux via the sodium pump) and 1 μCi $^{22}Na^+$ ± inhibitors and serum, as described in Results, were added to the cells and left for 4 min. The radioactive medium was then removed and the cells were rapidly washed, counted for radioactivity and estimated for protein content as described for $^{22}Na^+$ efflux estimation. The $^{22}Na^+$ uptake was expressed as μmol Na^+/g protein/min and calculated by the following equation:

$$\text{influx} = \frac{dRc/dt}{SA_{ex}}$$

where dRc/dt represents the uptake (cpm of $^{22}Na^+$ taken up per gram of protein per min).

Measurement of Na^+/Na^+ exchange in Human Erythrocytes

This method is based on that of Canessa et al. [12]. Briefly, 10 ml of fresh heparinized blood was washed × 3 (K^+ wash buffer–140 mM KCl, 0.15 mM $MgCl_2$, 10 mM MOPS, pH 7.4 with Tris-HCl). 2 ml of packed erythrocytes were resuspended in 10 ml of Na^+ loading solution (10 mM NaCl, 130 mM KCl, 50 mM sucrose, pH 7.4 with Tris-HCl) containing nystatin [30 μg/ml] and left at 4°C in the dark for 20 min. The inclusion of this Na^+-specific ionophore enabled rapid equilibration between intra- and extracellular Na^+. After spinning out the cells, a further 2 ml of Na^+ loading solution was added containing 20 μCi ^{22}Na and left for a further 20 min at 4°C. The nystatin was removed by washing the cells × 4 in 'nystatin-wash solution' (10 mM NaCl, 130 mM KCl, 50 mM sucrose, 1 mM K_2HPO_4, 10 mM glucose, 0.1% bovine serum albumin, pH 7.4 with Tris-HCl) followed by three washes in K^+ wash solution. The final pellet was adjusted to a hematocrit of 0.5. 200 μl of ^{22}Na loaded red cell suspension was added to 10 ml of 'efflux medium' [either Na^+ efflux medium (in mM): 150 NaCl, 0.15 $MgCl_2$, 0.1 ouabain, 10 glucose, 0.01 bumetanide, 10 MOPS, pH 7.4 with Tris-HCl, or K^+ efflux medium where 150 mM NaCl had been replaced by 150 mM KCl] and incubated at 37°C. At times 0, 30 and 60 min, triplicate 1-ml aliquots were removed, centrifuged and 800 μl of supernatant was counted for radioactivity and the rate of Na^+ efflux determined (mM Na^+/liter cells/hr). The value obtained in the absence of external Na^+ (i.e., in K^+ flux media) was subtracted from the value obtained in the presence of external Na^+ (i.e., in Na^+ efflux media) to give a value for Na^+/Na^+ exchange.

Isolation of Inhibitin

Inhibitin was prepared from the culture medium of H.Ep.2 cells as previously described [41]. Briefly, 10-ml aliquots of culture medium were injected onto a Waters C_{18} Sep-Pak cartridge which had been prewetted with 5 ml of MeCN containing 0.1% TFA. After washing the cartridge with 5 ml of 0.1% TFA, the inhibitin eluted with 30% MeCN containing 0.1% TFA. The eluent was evaporated to dryness under vacuum and purified further on two sequential reverse-phase HPLC steps using a hypersil ODS 5 μm column on a LDC CCM gradient system (Laboratory Data Control, Riviera Beach, FL). The solvent system employed was: phase A, 0.1% TFA in water and phase B, 0.1% TFA in 90% acetonitrile/10% water. Gradient elution over a period of 45 min at a flow rate of 1 ml/min from 1–30% phase B was used to develop the chromatogram after an initial isocratic elution for 5 min with 1% phase B. The fractions eluting at 30–36 min were pooled and further purified by chromatography on the same column processed in the presence of HFBA. Inhibitin was collected as a single sharp peak with a retention time of 28–29 min. Approximately 1–2 μg of inhibitin was obtained from 11 of culture medium. The molecular weight of inhibitin was estimated to be 2,600 daltons [40], so the concentration of inhibitin in the culture medium was $\simeq 1 \times 10^{-9}$ M. When used in the Na^+ transport assays inhibitin was reconstituted in a volume equivalent to one-tenth the original volume of culture medium from which it was isolated (final concentration of inhibitin $\simeq 1 \times 10^{-8}$ M).

Estimation of Cell Volume

The cell volume of fibroblasts and H.Ep.2 cells were estimated using a coulter counter (Coulter Electronics Ltd., Luton, Beds) that had been calibrated with latex beads of a known diameter (13.5 μm).

Statistical Methods

All data are presented as means ± 1 SD. Student's *t* test was used to determine significance between measurements in fibroblasts

Table 1. Total, ouabain-insensitive and ouabain-sensitive Na^+ efflux measured in fibroblasts and H.Ep.2 cells in HBSS ± 10% FCS[a]

Cell type	Na_i^+	+/− 10% FCS	Total		Ouabain insensitive		Ouabain sensitive	
			Rate constant per min	Absolute flux	Rate constant per min	Absolute flux	Rate constant per min	Absolute flux
Fibroblasts	0.043 ± 0.015	−	0.231 ± 0.034	9.92 ± 1.45	0.080 ± 0.009	3.44 ± 0.37	0.151 ± 0.031	6.48 ± 1.35
		+	0.785 ± 0.155	33.73 ± 6.65	0.306 ± 0.031	13.17 ± 1.33	0.478 ± 0.148	20.57 ± 6.37
Serum-stimulated Na^+ efflux			0.554 ± 0.151	23.81 ± 6.50	0.226 ± 0.039	9.73 ± 1.70	0.328 ± 0.140	14.08 ± 6.04
H.Ep.2 cells	0.075 ± 0.008	−	0.196 ± 0.031	15.50 ± 1.22	0.103 ± 0.008	7.90 ± 1.47	0.090 ± 0.009	6.90 ± 1.120
		+	0.547 ± 0.126	42.40 ± 13.00	0.312 ± 0.050	24.20 ± 3.80	0.290 ± 0.061	22.30 ± 4.10
Serum-stimulated Na^+ efflux			0.351 ± 0.113	30.50 ± 6.80	0.209 ± 0.049	16.10 ± 3.10	0.200 ± 0.058	15.10 ± 3.90

[a] Results are mean ± 1 SD for six separate estimations. Absolute flux = μmol Na^+/g protein/min.
Na_i^+ = intracellular Na^+ = μmol Na^+/mg protein

and H.Ep.2 cells, and between the effects of various inhibitors and serum.

Results

Breakdown of Na^+ Efflux into Active and Passive Effluxes in Fibroblasts and H.Ep. 2 cells

Na^+ efflux was measured in HBSS ± 0.5 mM ouabain ± 10% FCS in fibroblasts and H.Ep.2 cells, and the results are presented in Table 1. H.Ep.2 cells consistently showed higher values for intracellular Na^+ content with a mean of 0.075 ± 0.008 μmol Na^+/mg protein compared with that in fibroblasts (0.043 ± 0.015 μmol Na^+/mg protein; $P < 0.001$). In the absence of serum, H.Ep.2 cells showed significantly higher rates of total and passive Na^+ efflux ($P < 0.001$), but there was no significant difference in the active (ouabain-sensitive) Na^+ efflux between the two cell types. In the presence of 10% FCS, both the active and passive Na^+ effluxes increased. In fibroblasts the basal level of active Na^+ efflux, 6.48 ± 1.35 μmol Na^+/g protein/min, increased to 20.57 ± 6.37 μmol Na^+/g protein/min ($P < 0.005$) and the passive component of 3.44 ± 0.37 rose to 13.17 ± 1.33 μmol Na^+/g protein/min ($P < 0.001$). In H.Ep.2 cells the basal and serum stimulated values for the active components were 6.9 ± 1.12 and 22.3 ± 4.1 μmol Na^+/g protein/min ($P < 0.005$), respectively, while the passive increased from 7.9 ± 1.47 to 24.2 ± 3.8 μmol Na^+/g protein/min ($P < 0.005$) in the presence of 10% FCS. As described in Materials and Methods, the absolute flux rate is the product of the intracellular Na^+ content and the efflux rate constant. In order to establish whether this difference in Na^+ content (expressed as μmol Na^+/mg protein) was due to a lower protein content and/or larger cell volumes in H.Ep.2 cells, the number of cells representing 1 mg of cellular protein and the relative cell volumes were determined. Protein estimations carried out on known numbers of fibroblasts and H.Ep.2 cells showed that fibroblasts contained 4.9×10^{-4} μg protein/cell and H.Ep.2 cells contained 5.6×10^{-4} μg protein/cell. The relative cell volumes, estimated with a coulter counter, were 1670 and 1574 μm^3 in fibroblasts and H.Ep.2 cells, respectively. From these data the intracellular Na^+ concentration of fibroblasts was estimated at 12 mM and that of H.Ep.2 cells at 26 mM.

Breakdown of Passive Na^+ Transport in Fibroblasts and H.Ep.2 Cells

Na^+ fluxes were measured on the two cell types to quantify the proportion of passive (ouabain-insensitive) transport attributable to Na^+,K^+ cotransport (bumetanide-sensitive), Na^+/H^+ exchange (amiloride-sensitive) and Na^+/Na^+ exchange (measured as the difference in Na^+ efflux in 140 mM Na^+ and 14 mM Na^+ containing mediums). The measurements were made in the absence and presence of 10% FCS to establish which passive transport process was stimulated by serum. The results are shown in Table 2. In both cell types there were small components of both Na^+ influx and efflux that were sensitive to 0.1 mM bumetanide. These did not change significantly in the presence of 10% FCS or contribute to net flux. When amiloride (1 mM) was tested on fibroblasts in the absence of serum, there

Table 2. Breakdown of passive (ouabain-insensitive) Na^+ movement (results represent the average of four measurements)[a]

Cell type	Na^+ transport pathway	+/− 10% FCS	Influx	Efflux
			(moles Na^+/g protein/min)	
Fibroblasts	Passive	−	11.03 ± 2.37	3.20 ± 0.24
		+	35.40 ± 3.55	12.86 ± 1.44
	Bumetanide-sensitive Passive	−	0.67 ± 0.10	0.80 ± 0.30
		+	1.23 ± 0.91	1.52 ± 0.54
	Amiloride-sensitive Passive	−	1.40 ± 0.34	0.01 ± 0.09
		+	12.30 ± 4.34	1.35 ± 0.21
	External Na^+ Dependent passive	−	—	0.44 ± 0.07
		+	—	3.70 ± 0.50
H.Ep.2	Passive	−	19.97 ± 1.57	9.28 ± 1.00
		+	40.44 ± 4.28	29.26 ± 5.64
	Bumetanide-sensitive Passive	−	1.45 ± 0.48	2.14 ± 0.77
		+	2.63 ± 0.63	2.64 ± 0.49
	Amiloride-sensitive Passive	−	6.29 ± 0.80	1.98 ± 0.21
		+	7.96 ± 2.71	2.34 ± 0.33
	External Na^+ Dependent passive	−	—	2.20 ± 0.36
		+	—	11.45 ± 2.84

[a] Ouabain-insensitive Na^+ flux was measured in the presence of (i) 0.1 mM bumetanide, (ii) 1 mM amiloride, and (iii) 14 mM external Na^+. The fluxes under these conditions were subtracted from ouabain-insensitive Na^+ flux to obtain values for bumetanide-sensitive, amiloride-sensitive and external Na^+ dependent Na^+ fluxes.

was no change in the rates of either influx or efflux. However, when 10% FCS was present, an amiloride-sensitive rate of Na^+ influx was measured (12.3 ± 4.34 μmol Na^+/g protein/min) which was significantly greater ($P < 0.01$) than amiloride-sensitive efflux (1.35 ± 0.21 μmol Na^+/g protein/min), showing that net Na^+ influx was occurring via this pathway. For H.Ep.2 cells, even in the absence of serum, significant levels of amiloride-sensitive Na^+ flux were measured with the influx rate of 6.29 ± 0.80 μmol Na^+/g protein/min exceeding the efflux rate of 1.98 ± 0.21 μmol Na^+/g protein/min. In the presence of 10% FCS, there were small increases in amiloride-sensitive fluxes, with the influx rate still exceeding the efflux rate.

Na^+/Na^+ exchange was measured as the difference in Na^+ efflux in HBSS containing 14 or 140 mM Na^+ + 0.5 mM ouabain + 0.1 mM bumetanide ± 10% FCS. The Na^+ content of HBSS was replaced with equimolar amounts of choline chloride. 14 mM Na^+ was included in the serum-free experiments to compensate for the Na^+ content of FCS (estimated as 140 mM by flame photometry). This was done so that any differences in Na^+ efflux rates in the presence of FCS would be due to factors other than the 14 mM Na^+ contribution. It was decided not to dialyze FCS to remove the salt, in case any other small molecular weight nutrients might be lost that may contribute to the phenomenon of serum-stimulated Na^+ fluxes. Amiloride was not included in these experiments as there is evidence to suggest that Na^+/Na^+ exchange may be a different mode of the Na^+/H^+ exchanger [3, 6, 25, 50].

In the absence of FCS, fibroblasts and H.Ep.2 cells both had small components of Na^+/Na^+ exchange (0.44 ± 0.068 and 2.2 ± 0.36 μmol Na^+/g protein/min, respectively). In the presence of 10% FCS, this component was significantly increased in both cell types. In fibroblasts it rose from 0.44 ± 0.068 to 3.7 ± 0.5 μmol Na^+/g protein/min ($P < 0.005$), whereas in H.Ep.2 cells the basal rate of 2.2 ± 0.36 rose to 11.45 ± 2.84 μmol Na^+/g protein/min ($P < 0.01$).

Kinetic Study of Na^+/H^+ and Na^+/Na^+ Exchange

In order to investigate the interaction between Na^+/H^+ and Na^+/Na^+ exchanges, fibroblasts and H.Ep.2 cells were preincubated in HBSS + 1 mM ouabain to load the cells with high intracellular Na^+ concentrations (>100 mM). The reasons for adopting this procedure were twofold; first, to ensure sat-

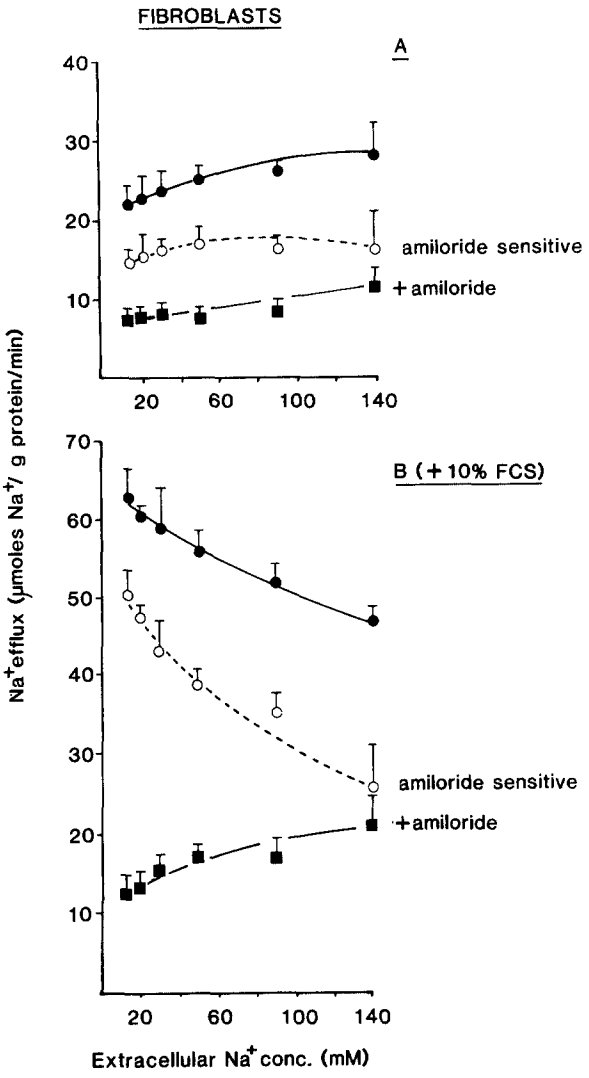

Fig. 1. Measurement of ouabain and bumetanide-insensitive Na^+ efflux as a function of extracellular Na^+ concentration in Na^+-loaded fibroblasts in (*A*) the absence and (*B*) the presence of 10% FCS. Fibroblasts were loaded with ^{22}Na in HBSS containing 1 mM ouabain. At the end of 2 hr incubation, the cells contained approximately 140 mM Na^+. Na^+ efflux was measured in ChCl-substituted HBSS (Na_o^+ concentration ranged from 14–140 mM Na^+)

uration of the internal sites so that intracellular Na^+ was not rate limiting and, secondly, to enable the construction of a steep outwardly directed Na^+ gradient (i.e., $Na_i^+ > Na_o^+$), which has been shown to cause reverse functioning of Na^+/H^+ exchange [9, 14, 35, 38, 59]. Ouabain- and bumetanide-insensitive Na^+ efflux was measured over a range of extracellular Na^+ concentrations (14–140 mM) in the absence and presence of 1 mM amiloride and 10% FCS, and the results are shown in Fig. 1 (fibroblasts) and Fig. 2 (H.Ep.2 cells).

In fibroblasts, in the absence of FCS (Fig. 1*A*), the rate of Na^+ efflux was not significantly altered with increasing extracellular Na^+ and approximately 80% of Na^+ efflux was amiloride sensitive. This indicates that high intracellular Na^+ promotes an amiloride-sensitive Na^+ efflux pathway, and at the external site of the transporter there appears to be equal affinity for H^+ and Na^+. In the presence of 10% FCS (Fig. 1*B*), Na^+ efflux was the highest at 14 mM external Na^+, when the concentration gradient was the steepest ($Na_i^+ > Na_o^+$) and 80% of this efflux was amiloride sensitive. This implies that factors present in serum can activate Na^+ efflux via an amiloride-sensitive pathway over and above the activation due to high intracellular Na^+ content. As the extracellular Na^+ concentration increased, the Na^+ efflux rate decreased, thus implying that serum factors have altered the kinetics of the transporter by increasing the affinity for H^+ and decreasing the affinity for Na^+ as the external substrate.

In Na^+-loaded H.Ep.2 cells in the absence of 10% FCS, the Na^+ efflux rate was partially amiloride sensitive and, as found with fibroblasts, the rate did not change significantly in response to raising the extracellular Na^+ concentration (Fig. 2*A*). In the presence of FCS, in contrast to fibroblasts, there was a marked increase in the rate of Na^+ efflux as the external Na^+ concentration increased (Fig. 2*B*), which suggests a predisposition of these Na^+-loaded cells to perform Na^+/Na^+ exchange in response to FCS. The concomitant decrease in amiloride-sensitive Na^+ efflux with increasing extracellular Na^+ could be a result of competitive inhibition [4, 21] or activation of Na^+/Na^+ exchange via an amiloride-insensitive transporter. The data from Fig. 2*B* is presented as a Lineweaver-Burk plot (inset Fig. 2*B*), which suggests that amiloride is a competitive inhibitor increasing the value of K_m from 20 to 91 mM Na^+, with no change for the V_{max} value of 62 μmol Na^+/g protein/min.

Although these experiments were not performed with the cells containing physiological concentration of intracellular Na^+, they clearly demonstrate that the kinetic parameters governing the flux of Na^+ via Na^+/H^+ and Na^+/Na^+ exchange are different in fibroblasts and H.Ep.2 cells.

Effect of Exogenous Inhibitin Na^+ Transport

The preceeding experiments were designed to characterize the Na^+ transport characteristics of fibroblasts (nonsecreter of inhibitin) and H.Ep.2 cells (inhibitin secreter), and the following differences were identified:

1) H.Ep.2 cells maintained higher intracellular Na^+

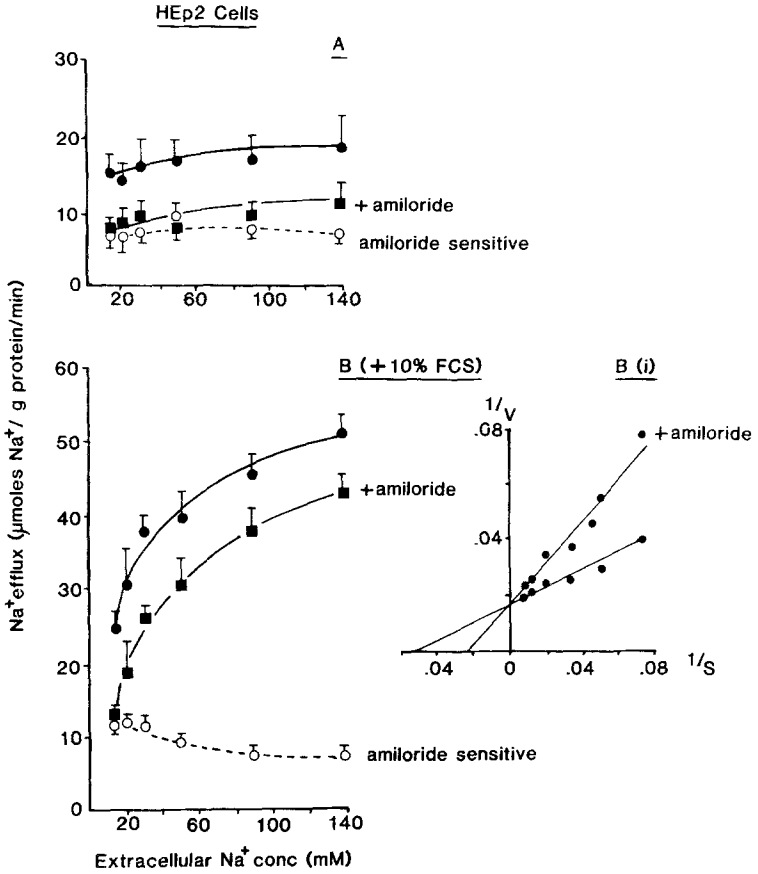

Fig. 2. Measurement of ouabain and bumetanide-insensitive Na^+ efflux as a function of external Na^+ concentration in Na^+-loaded H.Ep.2 cells in (*A*) the absence and (*B*) the presence of 10% FCS. H.Ep.2 cells were loaded with ^{22}Na in HBSS containing 1 mM ouabain. At the end of 2 hr incubation the cells contained approximately 100 mM Na^+. Na^+ efflux was measured in ChCl-substituted HBSS (Na^+ concentration ranged from 14–140 mM Na^+). Inset [*B(i)*] shows the data from *B* presented as a Lineweaver-Burk plot

concentration (26 mM) compared to fibroblasts (12 mM).

2) H.Ep.2 cells performed net Na^+ influx via an amiloride-sensitive pathway in the absence of FCS.
3) H.Ep.2 cells showed significantly higher levels of Na^+/Na^+ exchange in response to serum stimulation.
4) Under conditions dynamically poised to favor net Na^+ efflux against an imposed Na^+ gradient ($Na_i^+ > Na_o^+$), 10% FCS increased this downhill transport in fibroblasts, but not in H.Ep.2 cells.

To elucidate whether any of these differences in the regulation of Na^+ fluxes in H.Ep.2 cells could be attributable to the endogenous production of inhibitin, exogenous inhibitin was prepared (*see* Materials and Methods) and tested on the Na^+ transport characteristics of fibroblasts.

Effect of Inhibitin on the Intracellular Na^+ Concentration of Fibroblasts

The intracellular Na^+ concentration was estimated as previously described and the results are shown in Fig. 3. In the absence of inhibitin, the mean intracellular Na^+ concentration was 14 ± 2.2 mM Na^+, but in the presence of inhibitin this value was significantly increased to 19.4 ± 1.64 mM Na^+ ($P < 0.01$).

Effect of Inhibitin on Na^+ Transport in Fibroblasts

Inhibitin was tested on both Na^+ influx and Na^+ efflux in fibroblasts containing physiological levels of intracellular Na^+, and the measurements were performed in both the absence and presence of 10% FCS. There was no significant inhibitin-sensitive Na^+ flux either in the absence or presence of 10% FCS (*data not shown*).

As the characterization studies had revealed some fundamental differences between fibroblasts and H.Ep.2 cells in their response to a Na^+ load, inhibitin was tested on fibroblasts under these conditions and the results are shown in Fig. 4. In the absence of FCS, when Na^+ efflux was measured in 140 mM Na^+-containing HBSS, an inhibitin-sensitive component of 3.9 ± 0.52 μmol Na^+/g protein/min was obtained ($n = 6$, $P < 0.01$). In the presence of 10% FCS, this component increased 8.71 ± 4.68 μmol Na^+/g protein/min ($n = 4$, $P < 0.05$). How-

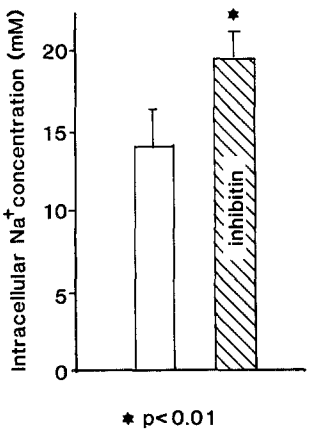

Fig. 3. The effect of exogenous inhibitin on the intracellular Na^+ concentration of fibroblasts. Dishes of fibroblasts were incubated for 2 hr in HBSS ± inhibitin + 0.5 μCi/ml ^{22}Na. The intracellular Na^+ content was estimated as described in Materials and Methods. The intracellular Na^+ concentration was calculated using the data that fibroblasts contain 4.9×10^{-4} μg protein/cell and have an intracellular volume of 1.67×10^{-9} ml. *$P < 0.01$

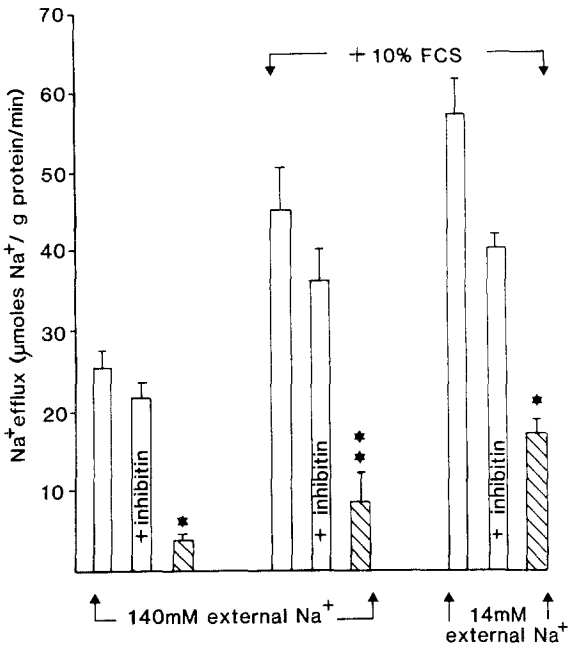

Fig. 4. The effect of inhibitin on ouabain and bumetanide-insensitive Na^+ efflux measured in fibroblasts loaded to contain high levels of intracellular Na^+ concentration (>100 mM). Measurements in the presence of inhibitin are shown and the hatched areas depict the inhibitin-sensitive fraction. The efflux was measured in the presence of 10% FCS where indicated and the efflux medium containing either 140 or 14 mM Na^+ is shown at the bottom of the graph

ever, when Na^+ efflux was measured into HBSS containing only 14 mM (the Na^+ contribution of 10% FCS), the inhibitin-sensitive component was significantly greater at 17.4 ± 1.5 μmol Na^+/g protein/min ($n = 3$, $P < 0.05$). Using inhibitin in combination with 1 mM amiloride under these conditions did not produce any further inhibition than found with amiloride alone (*data not shown*). These results strongly suggest that inhibitin plays a role in preventing Na^+ efflux via Na^+/H^+ exchange when working in the Na^+_{out} H^+_{in} mode.

Intracellular Na^+ Concentration, Na^+/H^+ and Na^+/Na^+ Exchange in Cultured Cells with Finite and Infinite Mortality

Apart from their inability to produce 'inhibitin,'' the fibroblasts differed from H.Ep.2 cells in three fundamental respects: fibroblasts are (i) mesenchymal cells, were grown from (ii) a normal tissue source (human foreskin) and (iii) have a finite life span. In contrast, H.Ep.2 cells have (i) epithelial morphology and originated from (ii) malignant tissue (human larynx carcinoma) and are (iii) immortal.

To investigate whether any of these three differences could explain the high intracellular Na^+ concentration of H.Ep.2 cells, a variety of other cell lines were studied. These included normal cells of both human (W138-embryonic lung) and animal (fetal rat hypothalamus) origins. A cell line that was immortal but exhibited contact-inhibited growth was included (MDCK cells). Other immortal cell lines chosen included cells isolated from normal tissue (T47D-human breast, L132-human lung) and cells from an abnormal mesenchymal source—a fibrosarcoma (HT-1080 cells). W138 V13 cells were W138 cells that had been transformed with SV-40 virus in vitro.

Intracellular Na^+ concentration was measured in all those cells as previously described for human foreskin fibroblasts and H.Ep.2 cells. Also measured was the rate of Na^+ efflux in Na^+-loaded cells into low (14 mM) and high (140 mM) Na^+-containing medium (+10% FCS). The results of these measurements are shown in Table 3. The intracellular Na^+ concentration of the cells with the 'transformed' phenotype, i.e., T47D, L132, HT-1080 and W138 VA13 were consistently higher than the two normal cell lines W138 and fetal rat brain cells. The best comparison is demonstrated in the normal W138 cells that were virally transformed, when Na^+ concentrations were 15 and 23 mM, respectively.

The immortal cell lines were consistent in demonstrating that when Na^+ loaded in the presence of 10% FCS, Na^+ efflux increased in response to high extracellular Na^+, indicating a kinetic preference for operating Na^+/Na^+ exchange. The reverse was true for the two normal cell lines studied, i.e., a

Table 3. Measurement of intracellular Na^+ concentration and Na^+ efflux in a range of normal and transformed cells[a]

Cell line	Life span	Origin	Morphology	Intracellular Na^+ concentration (mM)	Na^+ efflux from Na^+-loaded cells (μmol Na^+/g protein/min)			
					External Na^+ concentration		Effect of 140 mM external Na^+[b-a]	
					14 mM[a]	140 mM[b]	Stimulation[c]	Inhibition[d]
W.138	Finite	Human embryonic lung	Fibroblast	15	53 ± 2	47 ± 3		6 ± 1
Fetal rat brain	Finite	Fetal rat hypothalamus	Fibroblast	12	57 ± 8	45 ± 8		11 ± 2
MDCK	Immortal	Dog kidney	Epithelial	16	19 ± 2	23 ± 2	4 ± 0	
T 47 D	Immortal	Human breast	Epithelial	22	18 ± 2	42 ± 3	24 ± 4	
L 132	Immortal	Human Lung	Epithelial	21	45 ± 9	78 ± 10	33 ± 18	
HT-1080	Immortal	Human fibrosarcoma	Epithelial	18	28 ± 6	36 ± 4	12 ± 4	
W.138 VA13	Immortal	Human embryonic lung virally transformed with SV40	Epithelial	23	36 ± 7	67 ± 5	32 ± 9	

[a] Na^+ efflux was measured from Na^+-loaded cells into (a) 14 mM and (b) 140 mM Na^+-containing efflux medium (+10% FCS). These results were compared to estimate whether (c) stimulation or (d) inhibition of Na^+ efflux was associated with increasing extracellular Na^+ concentration.

decrease in Na^+ efflux in the 140 mM Na^+-containing medium.

From these studies it appears that high intracellular Na^+, and the kinetic preference of the Na^+/H^+ exchanger to perform Na^+/Na^+ exchange in response to a Na^+ load, appears to be a property of immortal cell lines regardless of their origin (i.e., cancerous or normal, mesenchymal or ectodermal origin) and whether they achieved immortality spontaneously (T47D, L132) or were virally transformed (W138 VA13).

Investigation for 'Inhibitin'-Like Activity

As a prelude to looking for 'inhibitin-like' activity in the culture medium of many cell lines growing with a 'transformed' phenotype, we chose to investigate W138 and W138 VA13 cells. These seemed ideal for comparison as they were different in only one respect, i.e., nontransformed and transformed.

'Inhibitin' had been originally isolated from the culture medium of HL60 and H.Ep.2 cells on its ability to inhibit Na^+/Na^+ exchange in human erythrocytes; we therefore decided to use this assay (as described in Materials and Methods) to monitor for 'inhibitin-like' activity in the culture medium conditioned with W138 and W138 VA13 cells. 500 ml of medium from both cell types were 'sep-pak' concentrated as described for H.Ep.2 cell medium (Materials and Methods). After evaporating to dryness, these concentrates were reconstituted in Na^+/Na^+ exchange assay medium to give a 5 × concentration factor. The rate of human erythrocyte Na^+/Na^+ exchange in the presence of W138 V13 medium was 0.041 ± 0.015 mM Na^+/liter cells/hr ($n = 3$), which was significantly lower than the value measured in the presence of W138 medium (0.089 ± 0.010 mM Na^+/liter cells/hr, $P < 0.01$).

Discussion

A variety of cultured malignant cells produce a peptidic Na^+ transport inhibitor (inhibitin) [40, 41]. The functional significance of this factor has remained unexplained. This study was undertaken to characterize Na^+ transport in normal human fibroblasts (nonproducers) and H.Ep.2 cells (inhibitin producers) and thereby investigate whether the intrinsic differences between the two cell types could be attributable to endogenous inhibitin.

Under basal conditions (i.e., physiological salt solution in the absence of FCS), the intracellular Na^+ content of H.Ep.2 cells was significantly higher than fibroblasts (Table 1), and converting those values to intracellular Na^+ concentration revealed an

even greater difference between the two cell types (i.e., 26 mM Na^+ for H.Ep.2 cells compared to 12 mM for fibroblasts). Increased intracellular Na^+ concentration has been known to be associated with neoplastic transformation [11, 55]. In the presence of 10% FCS both active and passive Na^+ efflux rates significantly increased in the two cell types, but, as found in the basal state, the Na^+ efflux rate was higher in H.Ep.2 cells due to a higher ouabain-insensitive component. The Na^+ content of a cell is governed by the ground permeability of the cell membrane, active Na^+ transport and passive carrier-mediated Na^+ transport that can achieve net fluxes. As active transport of the two cell types was not significantly different, a detailed study of passive Na^+ transport was undertaken to explore the reasons for a higher intracellular Na^+ concentration in H.Ep.2 cells. Bumetanide was used to quantify Na^+/K^+ cotransport. In both cell types under the experimental conditions used, this pathway did not contribute to net Na^+ movement and was not stimulated by 10% FCS. These results are in agreement with one report [45] though in conflict with a study using NIH 3T3 fibroblasts where this pathway was stimulated by serum [46]. Amiloride was used as a specific inhibitor of Na^+/H^+ exchange. Under basal conditions Na^+ transport in fibroblasts was amiloride insensitive. In the presence of 10% FCS, net Na^+ influx occurred via this pathway, which is in agreement with other reports [60, 61]. In contrast, H.Ep.2 cells were found to have a basal rate of net amiloride-sensitive Na^+ influx that was only slightly increased in the presence of 10% FCS.

There is evidence for the existence of a carrier-mediated Na^+/Na^+ exchange in red blood cells [17, 30, 31] and cultured cells [51]. This pathway was assessed as the difference in ouabain-insensitive and bumetanide-insensitive Na^+ efflux, measured in low Na^+ (14 mM) and high Na^+ (140 mM) containing efflux mediums. Our preliminary studies showed a K_m value of 22 mM Na^+ on the outside for this pathway (*unpublished data*). A small component was measurable in both cell types basally, and it increased significantly in the presence of 10% FCS. In fibroblasts, the basal rate of 0.44 ± 0.77 μmol Na^+/g protein/min rose to 3.7 ± 0.5 μmol Na^+/g protein/min, and in H.Ep.2 cells the rate of 2.2 ± 0.36 rose to 11.45 ± 2.84 μmol Na^+/g protein/min in the presence of serum.

The physiological significance of Na^+/Na^+ exchange has remained unknown. Recently, various investigators have proposed that the carrier protein of Na^+/Na^+ and Na^+/H^+ exchange may be one and the same [3, 6, 25], and the change of mode for each pathway may be governed by allosteric sites activated by growth factors that alter the affinity of the carrier protein to internal Na^+ and H^+ [3]. Na^+/H^+ exchange can operate in reverse (i.e., H^+ in and Na^+ out) when a steep $Na_i^+ > Na_o^+$ gradient is imposed [9, 14, 35]. To investigate this mode, fibroblasts and H.Ep.2 cells were loaded with high concentration of intracellular Na^+, and efflux was measured over a range of extracellular Na^+ concentrations. In the absence of FCS, no major differences between the two cells were observed and there was little change between Na^+ efflux measured in low Na^+ and high Na^+ containing mediums. This suggests that in the absence of FCS the intracellular Na^+ concentration controls the rate of efflux and there appears to be equal affinity for H^+ and Na^+ as external substrates. In the presence of 10% FCS, however, fibroblasts and H.Ep.2 cells showed an interesting difference in response to external Na^+. In fibroblasts, the highest rate of Na^+ efflux occurred when the Na^+ gradient was steepest and fell as the extracellular Na^+ concentration increased, suggesting a selective stimulation of Na^+/H^+ exchange (in the reverse mode). In contrast, H.Ep.2 cells under serum-stimulated conditions demonstrated their ability to conserve intracellular Na^+, since the efflux rate was lowest when the concentration gradient was steepest. As extracellular Na^+ concentration increased, Na^+ efflux increased. This suggests that H.Ep.2 cells have a modified Na^+/H^+ system, whereby the system will act in the $Na_{in}^+ H_{out}^+$ mode without growth factor activation but resists functioning in the $Na_{out}^+ H_{in}^+$ mode in response to serum when the intracellular Na^+ concentration is high. Under these conditions this pathway seems to operate preferentially as a Na^+/Na^+ exchanger. This interchange of Na^+/H^+ and Na^+/Na^+ enables H.Ep.2 cells to maintain a high pH and Na content inside. We extended these studies to a number of other cell lines and found that those showing 'transformed' characteristics in culture (whether of normal, neoplastic, epithelial or mesenchymal origin) had high intracellular Na^+ concentrations and modified Na^+/H^+ exchangers. We propose that inhibitin is responsible for this important change of mode in neoplastic and transformed cells. Evidence to support this proposed role of inhibitin was obtained when it was tested on fibroblasts under various conditions (Figs. 3 and 4). Inhibitin significantly raised the intracellular Na^+ concentration from 14 ± 2.4 mM Na^+ to 19 ± 1.5 mM Na^+ and showed a greater inhibition in Na^+-loaded, serum-stimulated fibroblasts in low Na^+ efflux medium, when Na^+/H^+ exchange would be expected to operate in the reverse mode. Although we have established that cell lines other than H.Ep.2 cells have the characteristics of this 'inhibitin' secreter, i.e., high intracellular Na^+ and modified Na^+/H^+ exchange, it would not be

justified to state at present that these characteristics are solely due to the endogenous production of this factor. However, it remains an interesting proposal which we hope to pursue.

Evidence that changes in the characteristics of Na^+/H^+ exchange are related to neoplasia is given in a recent report by Costa-Casnellie et al. [15], where the transporter was studied in HL-60 cells (human promyelocyte leukemic cell line) before and after inducing the cells to mature into granulocytes using dimethyl sulphoxide (Me_2SO). Initially, the rate of Na^+/H^+ increased, but after 3 days of Me_2SO treatment when 80% of the cells were producing superoxide, a property of mature granulocytes, Na^+/H^+ exchange dropped to 30% of control (untreated) cells. The matured cells had a decreased K_m for Na^+, and there appeared to be a shift in the affinity for intracellular H^+. It is worth pointing out that 'inhibitin' was originally isolated from the culture medium of HL.60 cells [40] and when HL.60 cells were matured using retinoic acid, the property of the conditioned medium to inhibit Na^+/Na^+ exchange in erythrocytes disappeared [42]. It has also been demonstrated that, following transformation of Chinese hamster embryo fibroblasts, there was an increase in intracellular pH and this was attributed to an alteration in the functioning of the Na^+/H^+ transporter whereby it maintained a higher 'set point,' resulting in lower concentrations of intracellular H^+ [44].

Previous characterization studies have shown that inhibitin specifically inhibits Na^+/Na^+ exchange in erythrocytes [39]. These experiments on fibroblasts do not support this mode of action in this more complex cell. However, human erythrocytes do not show serum activation of Na^+/Na^+ or Na^+/H^+ exchange (*unpublished observations*) and this pathway may represent a vestigial process in these specialized, nondividing cells and may not be subject to the same controls as replicating cells.

The reasons for H.Ep.2 cells and transformed cells, in general, maintaining high intracellular Na^+ concentration can only be surmised: elevated intracellular Na^+ can trigger the release of Ca^{2+} from intracellular bound pools [26], and changes in Ca^{2+} concentrations are implicated as important signals for cell proliferation [5, 8, 18, 34, 63, 64]. It is interesting to note that transformed 3T3 cells have elevated levels of intracellular Ca^{2+} compared to their normal counterparts [8]. The prevention of Na^+/H^+ exchange operating in the reverse mode (i.e., Na^+_{out} H^+_{in}) would also help to conserve intracellular alkalinization, which is thought to be permissive for cell proliferation [13, 53].

In conclusion, H.Ep.2 cells maintain higher intracellular Na^+ concentration compared to fibroblasts. Although equivalent levels of active Na^+ transport are found in the two cell types, passive Na^+ fluxes are higher in H.Ep.2 cells with net Na^+ influx occurring via an amiloride-sensitive pathway, which is activated in fibroblasts only in the presence of FCS. FCS also increases active Na^+ transport and Na^+/Na^+ exchange. Under conditions of elevated intracellular Na^+, Na^+ efflux is much higher in fibroblasts in Na^+-free medium than in H.Ep.2 cells and inhibitin inhibits this efflux. These studies suggest that inhibitin enables H.Ep.2 cells to maintain a high intracellular Na^+ content. Further studies are in progress to investigate the direct effects of inhibitin on pH_i and whether inhibitin is a marker for cell transformation.

References

1. Aickin, C.C., Thomas, R.C. 1977. An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *J. Physiol. (London)* **273:**295–316
2. Aiton, J.E., Chipperfield, A.R., Lamb, J.F., Ogden, P., Simmons, N.L. 1981. Occurrence of passive furosemide sensitive transmembrane potassium transport in cultured cells. *Biochim. Biophys. Acta* **646:**389–398
3. Aronson, P.S., Nee, J., Suhm, M.A. 1982. Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillus membrane vesicles. *Nature (London)* **299:**161–163
4. Aronson, P.S., Suhm, M.A., Nee, J. 1983. Interaction of external H^+ with the Na^+ H^+ exchanger in renal microvillus membrane vesicles. *J. Biol. Chem.* **258:**6767–6771
5. Berridge, M.J. 1975. Control of cell division of unifying hypothesis. *J. Cyclic Nucle. Res.* **1:**305–320
6. Betz, A.L. 1983. Sodium transport in capillaries isolated from rat brain. *J. Neurochem.* **41:**1150–1157
7. Bourne, H., Rozengurt, E. 1976. An 18,000 molecular weight polypeptide induces early events and stimulates DNA synthesis in cultured cells. *Proc. Natl. Acad. Sci. USA* **73:**4555–4559
8. Boynton, A.L., Whitfield, J.F., Isaacs, R.J., Morton, H.J. 1974. Control of 3T3 cell proliferation by calcium. *In Vitro* **10:**12–17
9. Burnham, C., Munzesheimer, C., Rabon, E., Sachs, G. 1982. Ion pathways in renal brush border membranes. *Biochim. Biophys. Acta* **685:**260–272
10. Busa, W.B., Nuccitelli, R. 1984. Metabolic regulation via intracellular pH. *Am. J. Physiol.* **246:***R*409–*R*438
11. Cameron, I.L., Smith, N.K.R., Pool, T.B., Sparks, R.L. 1980. Intracellular concentration of sodium and other elements as related to mitogenesis and oncogenesis in vivo. *Cancer Res.* **40:**1493–1500
12. Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. 1986. Modes of operation and variable stoichiometry of the furosemide-sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.* **87:**113–142
13. Cassel, D., Rothenberg, P., Zhuag, Y.X., Dauel, T.F., Glaser, L. 1983. Platelet derived growth factor stimulates Na^+ H^+ exchange and induces cytoplasmic alkalinization in NR6 cells. *Proc. Natl. Acad. Sci. USA* **80:**6224–6228

14. Cohen, D.E., Hruska, K.A., Klahr, S., Hammerman, M.R. 1982. Increased Na^+ H^+ exchange in brush border vesicles from dogs with renal failure. *Am. J. Physiol.* **242:***F*293–*F*299
15. Costa-Casnellie, M.R., Segal, G.B., Cragoe, E.J., Jr., Lichtman, M.A. 1987. Characterisation of the Na^+/H^+ exchanger during maturation of HL.60 cells induced by dimethyl sulfoxide. *J. Biol. Chem.* **262:**9093–9097
16. Crane, R.K. 1964. Uphill outflow of sugar from intestinal epithelial cells induced by reversal of the Na^+ gradient: Its significance for the mechanism of Na^+ dependent active transport. *Biochim. Biophys. Res. Commun.* **17:**481–485
17. Dunn, M.J. 1970. The effects of transport inhibitors on sodium outflux and influx in red blood cells: Evidence for exchange diffusion. *J. Clin. Invest.* **49:**1804–1814
18. Durham, A.C.H., Walton, J.M. 1982. Calcium ions and the control of proliferation in normal and cancer cells. *Biosci. Rep.* **2:**15–30
19. Geck, P., Pictrzyk, C., Burckhardt, B.C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of Na, K and Cl in Ehrlich cells. *Biochim. Biophys. Acta* **600:**432–447
20. Glynn, I.M. 1956. Sodium and potassium movements in human red cells. *J. Physiol. (London)* **134:**278–310
21. Grinstein, S., Cohen, S., Rothstein, A. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J. Gen. Physiol.* **83:**341–369
22. Kasarov, L.B., Friedman, H. 1974. Enhanced Na^+ K^+-activated adenosine triphosphatase activity in transformed fibroblasts. *Cancer Res.* **34:**1862–1865
23. Kashiwagura, T., Deutsch, C.J., Taylor, J., Erecinska, M., Wilson, D.F. 1984. Dependence of gluconeogenesis, urea synthesis and energy metabolism of hepatocytes on intracellular pH. *J. Biol. Chem.* **259:**237–243
24. Kimelberg, H.K., Mayhew, E. 1975. Increased ouabain-sensitive $^{86}Rb^+$ uptake and sodium and potassium ion-activated adenosine triphosphatase activity in transformed cell lines. *J. Biol. Chem.* **250:**100–104
25. Kinsella, J.L., Aronson, P.S. 1981. Interaction of NH_4^+ and Li^+ with the renal microvillus membrane Na^+ H^+ exchanger. *Am. J. Physiol.* **241:***C*220–*C*226
26. Lowe, D.A., Richardson, B.P., Taylor, P., Donatsch, P. 1976. Increasing intracellular sodium triggers calcium release from bound pools. *Nature (London)* **260:**337–338
27. Lowry, O.H., Roseborough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
28. Lubin, M. 1967. Intracellular potassium and macromolecular synthesis in mammalian cells. *Nature (London)* **213:**451–453
29. Lubin, M., Ennis, H.L. 1964. On the role of intracellular potassium in protein synthesis. *Biochim. Biophys. Acta* **80:**614–631
30. Lubowitz, H. 1972. Exchange diffusion in human red blood cells. *Proc. Soc. Exp. Biol. Med.* **140:**153–156
31. Lubowitz, H., Whittam, R. 1969. Ion movements in human red cells independent of the sodium pump. *J. Physiol. (London)* **202:**111–131
32. McRoberts, J.A., Erlinger, S., Rindler, M.J., Saier, M.J., Jr. 1982. Furosemide-sensitive salt transport in the Madin-Darby canine kidney cell line. *J. Biol. Chem.* **257:**2260–2266
33. Mendoza, S.A., Wigglesworth, N.M., Pohjanpelto, P., Rozengurt, E. 1980. Na entry and Na-K pump activity in murine, hamster and human cells: Effect of monensin, serum, platelet extract and viral transformation. *J. Cell. Physiol.* **103:**17–27
34. Metcalfe, J.C., Pozzan, T., Smith, G.A., Heskith, T.R. 1980. A calcium hypothesis for the control of cell growth. *Biochem. Soc. Symp.* **45:**1–26
35. Moolenaar, W.H., Boonstra, J., Van der Saag, P.T., Laat, S.W. de 1981. Sodium/proton exchange in mouse neuroblastoma cells. *J. Biol. Chem.* **256:**12883–12887
36. Moolenaar, W.H., Mummery, C.L., Van der Saag, P.T., Laat, S.W. de 1981. Rapid ionic events and the initiation of growth in serum stimulated neuroblastoma cells. *Cell* **23:**789–798
37. Moolenaar, W.H., Yarden, Y., Laat, S.W. de, Schlessinger, J. 1982. Epidermal growth factor induces electrically silent Na^+ influx in human fibroblasts. *J. Biol. Chem.* **257:**8502–8506
38. Moore, R.D. 1981. Stimulation of Na : H exchange by insulin. *Biophys. J.* **33:**203–210
39. Morgan, K., Brown, R.C., Spurlock, G., Southgate, K., Mir, M.A. 1986. Inhibitin: A specific inhibitor of sodium/sodium exchange in erythrocytes. *J. Clin. Invest.* **77:**538–544
40. Morgan, K., Mir, M.A. 1984. Isolation of a sodium transport inhibitory factor, inhibitin, from cultured leukemic promyelocytes. *J. Clin. Invest.* **74:**1132–1142
41. Morgan, K., Spurlock, G., Brown, R.C., Mir, M.A. 1987. Release of a sodium transport inhibitor (inhibitin) from cultured human cancer cells. *Cancer Res.* **45:**6095–6100
42. Morgan, K., Spurlock, G., Mir, M.A. 1985. Leukaemic promyelocytes and normal bone marrow cells release a passive sodium transport modifier (inhibitin). *Clin. Sci.* **68:**365–368
43. Murer, H., Hopfer, U., Kinne, R. 1976. Sodium/proton antiport in brush-border membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* **154:**597–604
44. Ober, S.S., Pardee, A.B. 1987. Intracellular pH is increased after transformation of Chinese hamster embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **84:**2766–2770
45. Owen, N.E., Prastein, M.L. 1985. Na/K/Cl cotransport in cultured human fibroblasts. *J. Biol. Chem.* **260:**1445–1451
46. Panet, R. 1985. Serum induced net K^+ influx performed by the diuretic-sensitive transport system in quiescent NIH 3T3 mouse fibroblasts. *Biochim. Biophys. Acta* **813:**141–144
47. Post, R.L., Jolly, P.C. 1957. The linkage of sodium, potassium and ammonium active transport across the human erythrocyte membrane. *Biochim. Biophys. Acta* **25:**118–128
48. Pouyssegur, J., Chambard, J.C., Franchi, A., Paris, S., Obberghen-Schilling, E. van 1982. Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: Coupling to ribosomal protein S6 phosphorylation. *Proc. Natl. Acad. Sci. USA* **79:**3935–3939
49. Rindler, M.J., McRoberts, J.A., Saier, M.H., Jr. 1982. (Na^+, K^+) cotransport in MDCK cell lines. *J. Biol. Chem.* **257:**2254–2259
50. Rindler, M.J., Saier, M.H., Jr. 1981. Evidence for Na^+/H^+ antiport in cultured dog kidney cells (MDCK). *J. Biol. Chem.* **256:**10820–10825
51. Rindler, M.J., Taub, M., Saier, M.H., Jr. 1979. Uptake of $^{22}Na^+$ by cultured dog kidney cells (MDCK). *J. Biol. Chem.* **254:**11431–11439
52. Ross, R., Glosmet, J., Kariya, B., Harker, L. 1974. Platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc. Natl. Acad. Sci. USA* **71:**1207–1210
53. Rothenberg, P., Glaser, L., Schlesinger, P., Cassel, D. 1983. Activation of Na^+/H^+ exchange by epidermal growth factor elevates intracellular pH in A431 cells. *J. Biol. Chem.* **258:**12644–12653

54. Rozengurt, E., Heppel, L.A. 1975. Serum rapidly stimulates ouabain-sensitive $^{86}Rb^+$ influx in quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA* **72:**4492–4495
55. Shen, S.S., Hamamoto, S.T., Bern, H.A., Steinhardt, R.A. 1978. Alteration of sodium transport in mouse mammary epithelium associated with neoplastic transformation. *Cancer Res.* **38:**1356–1361
56. Smith, G.L. 1977. Increased ouabain-sensitive ^{86}rubidium uptake after mitogenic stimulation of quiescent chicken embryo fibroblasts with purified multiplication-stimulating activity. *J. Cell Biol.* **73:**761–767
57. Smith, J.B., Rozengurt, E. 1978. Serum stimulates the Na^+K^+ pump by quiescent fibroblasts by increasing Na^+ entry. *Proc. Natl. Acad. Sci. USA* **75:**5560–5564
58. Spurlock, G., Morgan, K., Mir, M.A. 1985. A reproducible procedure for measuring sodium transport in cultured human fibroblasts from normal and obese donors. *Clin. Chim. Acta* **153:**225–232
59. Spurlock, G., Morgan, K., Mir, M.A. 1986. Serum stimulation of sodium transport in human fibroblasts containing low and high levels of intracellular sodium. *J. Membrane Biol.* **92:**163–170
60. Villereal, M.L. 1981. Sodium fluxes in human fibroblasts: Effects of serum, Ca^{2+} and amiloride. *J. Cell. Physiol.* **107:**359–369
61. Villereal, M.L. 1981. Sodium fluxes in human fibroblasts: Kinetics of serum-dependent and serum-independent pathways. *J. Cell. Physiol.* **108:**251–259
62. Wheeler, K.P., Christensen, H.N. 1967. Role of Na^+ in the transport of amino acids in rabbit red cells. *J. Biol. Chem.* **242:**3782–3788
63. Whitfield, J.F., Boynton, A.L., MacManus, J.P., Rixon, R.H., Sikorska, M., Tsang, B., Walker, P.R., Swierenga, S.H.H. 1981. The roles of calcium and cyclic AMP in cell proliferation. *Ann. N.Y. Acad. Sci.* **339:**216–240
64. Whitfield, J.R., MacManus, J.P., Rixon, R.H., Boynton, A.C., Youdale, T., Swierenga, S.H.H. 1976. The possible control of cell proliferation by the interplay of calcium ions and cyclic nucleotides. *In Vitro* **12:**1–18
65. Wiley, J.S., Cooper, R.A. 1974. A frusemide-sensitive cotransport of sodium plus potassium in the human red cell. *J. Clin. Invest.* **53:**745–755

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