Activation of the Na⁺/H⁺ antiport is not required for epidermal growth factor-dependent gene expression, growth inhibition or proliferation in human breast cancer cells

Jon G. CHURCH,*† Gordon B. MILLS† and Ronald N. BUICK*

*Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9, and †Oncology Research, Toronto General Hospital, 200 Elizabeth Street, Toronto, Ontario, Canada M5G 2C4

Mitogen interaction with specific receptors in many cell types leads to activation of the Na⁺/H⁺ antiport and a resultant cytoplasmic alkalinization. Since amiloride inhibits both Na⁺/H⁺ exchange and cell proliferation, it has been hypothesized that activation of the antiport is an obligatory requirement for mitogenesis. However, concentrations of amiloride which inhibit the antiport also inhibit other cellular processes, including protein synthesis and phosphorylation. We have used an epidermal growth factor (EGF) receptor gene-amplified human breast cancer cell line, the growth of which is inhibited by high levels of EGF in culture (MDA-468) and a variant, the growth of which is stimulated by EGF (MDA-468-S4), along with two potent amiloride analogues to examine whether activation of the Na⁺/H⁺ antiport and cytoplasmic alkalinization is necessary for both EGF-dependent effects to occur. At concentrations of the amiloride analogues which block Na⁺/H⁺ exchange in both cell types by 76–98 %, the EGF-dependent alterations in [³H]thymidine incorporation or induction in *c-myc* or *c-fos* gene transcription were unaltered. These results were confirmed by a lack of effect of the amiloride analogues on both the growth-stimulatory and growth-inhibitory effects on EGF in an anchorage-independent growth assay. Similarly, in pH-altered media that prevented normal cytoplasmic alkalinization, the response of both MDA-468 and MDA-468-S4 to EGF activation was unaltered. In addition, activation of the Na⁺/H⁺ antiport alone was not sufficient to induce *c-myc* and *c-fos* transcription in either cell type. Taken together, these data suggest that neither the Na⁺/H⁺ antiport nor cytoplasmic alkalinization are necessary or sufficient for either EGF-dependent growth stimulation or growth inhibition in MDA-468 human breast cancer cells.

INTRODUCTION

Growth factors are polypeptide hormones that induce cell division in their target cells. They initiate their action by binding to specific cell-surface receptors and activating a cascade of rapid biochemical and physiological changes in the cell, which ultimately lead to DNA replication and cell proliferation. The early consequences of growth factor–receptor interaction include protein phosphorylation [1–3], a transient rise in cytoplasmic free calcium [4, 5], the temporary expression of several genes, including the nuclear-located proto-oncogene products *c-myc* and *c-fos* [6–8], and a stimulation of monovalent ion transport across the plasma membrane [9]. Of the known ionic transport changes in growth factor-stimulated cells, the activation of amiloride-sensitive Na⁺/H⁺ exchange has been widely examined [10, 11]. This electroneutral exchange of Na⁺ and H⁺ is mediated by the Na⁺/H⁺ antiport and is activated following the binding of a variety of growth factors, including insulin, thrombin, platelet-derived growth factor, interleukin-2 and epidermal growth factor (EGF), to their high affinity receptors [10–12]. The exchange of H⁺ for Na⁺ leads to both an intracellular alkalinization and an increase

in intracellular Na⁺ concentration [9]. Amiloride, a potassium-sparing diuretic, is a potent inhibitor of both Na⁺/H⁺ exchange and cell proliferation induced by mitogenic growth factors. This has led to suggestions that activation of Na⁺/H⁺ exchange, the resultant cytoplasmic alkalinization and/or increased Na⁺ concentration intracellularly may play a crucial role in the initiation of proliferation [9–14]. However, it is now known that amiloride may also affect cell proliferation by inhibition of protein phosphorylation [15], protein kinase C [16], or protein synthesis [17, 18]. A series of amiloride analogues have been synthesized [19], which have been demonstrated to have a much higher affinity for the Na⁺/H⁺ antiport than does the parent compound and the use of lower effective concentrations has been reported to decrease non-specific toxicity [14, 19].

In recent studies, we have reported that a human breast cancer cell line, MDA-468, has an amplified EGF-receptor gene and consequently a very high number of EGF receptors [20]. In supra-physiological concentrations of EGF, growth of MDA-468 is inhibited [20, 21], as has been reported for another EGF-receptor-gene-amplified cell line, A431 [22]. We used that fact to generate MDA-468 variants which survive in high EGF

Abbreviations used: EGF, epidermal growth factor; FCS, foetal-calf serum; BCECF, 2',7'-biscarboxyethyl-5,6-carboxyfluorescein; EPA, 5-(*N*-ethyl-*N*-propylamino)amiloride; EPPA, 5-[*N*-ethyl-*N*-(2-propenyl)amino] amiloride; pH_i, intracellular pH; pH_o, extracellular pH; [Na⁺]_i, intracellular Na⁺ concentration.

† To whom correspondence should be addressed.

concentrations and were subsequently found to have lost their gene amplification [21]. One variant cell line, MDA-468-S4, was found to show an absolute requirement for the addition of exogenous EGF for growth in anchorage-independent conditions. MDA-468 and MDA-468-S4, therefore, are closely related, differing primarily in the level of EGF-receptor expression, but respond differently to EGF in tissue culture.

In this paper, using both the potent amiloride analogues and the two cell lines which demonstrate opposite proliferative responses to EGF, we have examined the role of the Na^+/H^+ antiport and the resultant cytoplasmic alkalinization in the actions of EGF. Our data demonstrate that although activation of the antiport and cytoplasmic alkalinization take place in both cell types in response to EGF, it is not a prerequisite for augmented gene expression, growth inhibition or growth stimulation.

MATERIALS AND METHODS

Materials

Leibowitz (L-15), a nominally bicarbonate-free medium, was obtained from Gibco/BRL (Burlington, Ontario, Canada) and foetal-calf serum (FCS) was from Bocknek-British Drug House Chemicals (Toronto, Ontario, Canada). [^3H]Thymidine ($1.5\text{--}2.2\text{ TBq} \cdot \text{mmol}^{-1}$) and [^{32}P]dCTP ($110\text{ TBq} \cdot \text{mmol}^{-1}$), were from Amersham (Oakville, Ontario, Canada). 2',7'-Biscarboxyethyl-5,5-carboxyfluorescein (BCECF) acetoxymethyl ester was purchased from Molecular Probes (Eugene, Oregon, U.S.A.). The two amiloride analogues were a gift from Dr. E. J. Cragoe, Jr., Merck, Sharp and Dohme Laboratories (West Point, PA, U.S.A.) and were synthesized as described in ref. [19]. Each of the substituted amiloride analogues, 5-(*N*-ethyl-*N*-propylamino)amiloride (EPA), and 5-[*N*-ethyl-*N*-(2-propenyl)amino] amiloride (EPPA) were dissolved in dimethyl sulphoxide and diluted in medium immediately before use. Zetabind nylon membranes were purchased from AMF/Cuno (Meriden, CT, U.S.A.) EGF was obtained from Collaborative Research (Bedford, MA, U.S.A.).

Cell culture

Cell line MDA-468 was derived from a human breast tumour [23,24]. The variant, MDA-468-S4, was generated by this laboratory as reported in ref. [21]. Both cell lines were routinely cultured in nominally HCO_3^- -free L-15 medium, supplemented with 10% (v/v) FCS.

Cytoplasmic pH (pH_i) determinations

For fluorescence measurements $(1\text{--}10) \times 10^6 \cdot \text{ml}^{-1}$ cells were incubated with BCECF acetoxymethyl ester ($2.5\text{ }\mu\text{g} \cdot \text{ml}^{-1}$) in Buffer A (140 mM-NaCl/3 mM-KCl/1 mM-MgCl₂ / 10 mM-glucose / 1 mM-CaCl₂ / 20 mM-Hepes, pH 7.23) for 20–30 min [12,25]. The cells were washed three times in Buffer A and then resuspended in nominally HCO_3^- -free L-15 medium. Just before assay, the cells were washed and resuspended in Buffer A. The fluorescence emission was measured in a heated cuvette at 37 °C in a Hitachi model F4000 fluorescence spectrophotometer with excitation at 495 nm and emission at 526 nm. Calibration was obtained by incubation of cells in K^+ medium (141 mM-KCl/1 mM-CaCl₂/1 mM-MgCl₂/10 mM-glucose/20 mM-Hepes, pH 7.2) with $2\text{ }\mu\text{g}$ of nigericin $\cdot \text{ml}^{-1}$ (Calbiochem-Behring, San Diego, CA, U.S.A.) and titration, with aliquots of 1 M-Tris or

1 M-Mes [26]. Cell viability, as measured by the ability to maintain a Na^+ gradient, was measured by treating the cells in the assay with $5\text{ }\mu\text{M}$ -monensin [12,27].

All pH_i measurements with BCECF were performed in Buffer A. This nominally HCO_3^- -free medium was used to maximize changes in pH_i which were induced by the various agents tested, and to minimize cell volume changes which might alter the fluorescent signal, independently of pH changes.

Activation of the Na^+/H^+ antiport

Activity was measured after treating serum-starved cells (48 h) with EGF or after hyper-osmotic stress, procedures known to activate the antiport [10, 11, 28]. Briefly, BCECF-loaded cells $[(1\text{--}10) \times 10^6 \cdot \text{ml}^{-1}]$ were suspended in 1 ml of Buffer A and baseline fluorescence was determined. The amiloride analogue to be tested was then added, and the cells stimulated by the addition of EGF (10^{-8} M) or increased osmolarity by the addition of *N*-methylglucamine (140 mM). Again, monensin, an exogenous Na^+/H^+ exchanger, was used to verify cell integrity and the sensitivity of the assay.

Effect of EGF and amiloride analogues on anchorage-independent growth

Cells (10^4) were plated in triplicate on 35 mm plates in a two-layer system of agar (w/v, 0.5% bottom, 0.3% top layer) in nominally HCO_3^- -free L-15 medium supplemented with 10% (v/v) FCS and EGF and/or the amiloride analogues EPA or EPPA, at the concentrations noted. Plates were then incubated for 18 days at 37 °C and then analysed for colony formation (> 60 cells) by inverted microscopy.

EGF-dependent [^3H]thymidine incorporation

MDA-468 and MDA-468-S4 cells ($10^6/\text{plate}$) were grown on triplicate 35 mm plates in nominally HCO_3^- -free L-15 medium and 10% (v/v) FCS. After a 24 h equilibration period, MDA-468-S4 was serum-starved and both cell lines were treated with the indicated concentrations of amiloride analogues or medium of altered pH for an additional 48 h. [^3H]Thymidine ($5\text{ }\mu\text{Ci} \cdot \text{ml}^{-1}$) and EGF (10^{-8} M) were then added and cells incubated for 6 h. Incorporation was stopped by replacement with unlabelled, nominally HCO_3^- -free L-15 medium and [^3H]thymidine uptake was determined by scintillation counting after an 18 h chase.

Isolation of RNA and RNA blotting

Total RNA was isolated by guanidine isothiocyanate solubilization and centrifugation over a CsCl cushion [29]. Poly(A)⁺ RNA ($5\text{ }\mu\text{g}$), purified by passage over oligo(dT)-cellulose, was denatured with glyoxal and dimethyl sulphoxide and electrophoresis was performed in a 1.1% (w/v) agarose gel. The RNA was then transferred to a Zetabind filter and hybridized to a radiolabelled probe using high stringency conditions. Human 28 S and 18 S rRNAs were used as markers.

Probes

For *c-myc* analysis, the plasmid pMC41 RC, containing the entire third exon of the human *c-myc* gene was used [30]. For *c-fos* analysis the *p-fos-1* probe, derived from *v-fos*, was utilized [31]. Probes were ^{32}P -nick-translated using [^{32}P]dCTP as described [32].

RESULTS

Inhibition of the Na⁺/H⁺ antiport by amiloride and amiloride analogues

The ionic correlates of the stimulation of MDA-468 by EGF (10^{-8} M) are shown in Fig. 1. As reported elsewhere [10, 11], addition of EGF to cells suspended in medium of physiological Na⁺ concentration led to an intracellular alkalization of approx. 0.15 pH units and equilibrated within 8 min (Fig. 1). The binding of EGF to its receptor leads to an increase in pH_i through activation of the Na⁺/H⁺ antiport in MDA-468 cells. This conclusion is supported by the fact that EGF-induced alkalization can be attenuated by 100 μ M-amiloride, if added after EGF (Fig. 1), or completely blocked if the same concentration of amiloride is added just before EGF (results not shown). The response of MDA-468-S4 cells to both EGF and amiloride was identical with that shown in Fig. 1 for MDA-468 (results not shown). Because these studies were conducted in nominally HCO₃⁻-free Buffer A, the increase in pH_i could not have been the result of HCO₃⁻ moving into the cell either in exchange for Cl⁻ or by co-transport with Na⁺ [33, 34].

Since the electroneutral exchange of Na⁺ and H⁺ has been attributed to the Na⁺/H⁺ antiport [9–11] and can be blocked by the K⁺-sparing diuretic, amiloride, it was necessary to measure the potency of the amiloride analogues, EPA and EPPA, on MDA-468 and MDA-468-S4 cells. Because the Na⁺/H⁺ antiport is essentially quiescent in unstimulated cells, the activation of ionic exchange was measured after hyper-osmotic stress [28]. *N*-Methylglucamine (140 mM) was routinely used to activate Na⁺/H⁺ exchange in these cells, rather than EGF. As Figs. 1 and 2 will attest, *N*-methylglucamine offered the combined advantage of a faster equilibration with a higher level of pH_i compared with EGF at the supraphysiological levels (10^{-8} M) used in these experiments. However, like amiloride, EPA and EPPA were both able to block EGF-induced intracellular alkalization with a concentration response not unlike that which inhibits *N*-methylglucamine (results not shown). In addition, *N*-methylglucamine induces a cytoplasmic alkalization that is also sensitive to low concentrations of the amiloride analogues EPA and EPPA (Fig. 2a). These analogues were used in this study to allow us to

Fig. 1. Effect of EGF on pH_i of MDA-468 cells

MDA-468 cells were loaded with BCECF as described in the Materials and methods section. Cells (1×10^6) were washed and suspended in a cuvette in Buffer A medium. EGF (10^{-8} M), amiloride (100 μ M) and monensin (5 μ M) were added where indicated. The pH was calibrated as described in the Materials and methods section in nigericin-permeabilized cells with Mes and Tris.

Fig. 2. Inhibition of the Na⁺/H⁺ antiport in MDA-468 cells by amiloride analogues

MDA-468 cells were loaded with BCECF as described in the Materials and methods section. Cells (2×10^6) were washed and suspended in Buffer A in a cuvette and the baseline determined. Cytoplasmic alkalization was induced by the addition of *N*-methylglucamine (NMG) (140 mM) as shown. Concentration of the amiloride analogues effective in blocking the hyper-osmotic stress-induced increase in pH_i are as follows: (b) and (c) 0.1 and 1.0 μ M-EPA, respectively; (d) and (e) 0.1 and 1.0 μ M-EPPA, respectively. Monensin was added to establish the integrity of the cell membrane; shown only in (a). The system was calibrated following nigericin-permeabilization as outlined previously.

utilize lower concentrations that circumvent non-specific effects, but still inhibit the antiport [12, 14, 19]. As illustrated in Fig. 2, 1.0 μ M-EPA inhibited the steady-state magnitude of cytoplasmic alkalization owing to the hyper-osmotic stress induced by 140 mM-*N*-methylglucamine in MDA-468 cells by 76 % (Fig. 2c), while the same concentration of EPPA blocked the rise in pH_i by 98 % (Fig. 2e). Experiments in MDA-468-S4 cells, performed under the same conditions, yielded identical results, both in response to *N*-methylglucamine and to the amiloride analogues (results not shown).

The role of the Na⁺/H⁺ antiport and increased pH_i on EGF-dependent growth inhibition, growth stimulation and biochemical correlates

MDA-468 cells grow in anchorage-independent culture in the presence of 10 % (v/v) FCS but without exogenous EGF. The addition of supraphysiological concentrations of EGF (10^{-8} M) to such cultures results in a significant inhibition in colony formation (Table 1). In contrast, MDA-468-S4 cells are dependent on exogenous EGF to grow in anchorage-independent culture (Table 1). Therefore, we tested whether the opposing actions of EGF on the two cell types in agar culture required stimulation of the Na⁺/H⁺ antiport. As illustrated in Table 1, at concentrations of both EPA and EPPA that resulted in inhibition of the antiport, there was little alteration in the EGF-dependent changes in anchorage-independent

Table 1. Concentration-dependent effect of amiloride analogues on EGF-dependent changes in agar plating efficiency

Growth studies were carried out on MDA-468 and MDA-468-S4 cells (10^4 /plate) in agar containing EGF (10^{-8} M) and/or the various concentrations of amiloride analogues (EPA or EPPA) as described in the Materials and methods section and as shown in the Table. Data represent plating efficiency ($10^2 \times$ number of colonies/ 10^4 cells $\pm$ S.E.M.; $n = 3$), where a colony is defined as > 60 cells.

Conditions	Plating efficiency ($10^2 \times$ no. of colonies/ 10^4 cells)	
	MDA-468	MDA-468-S4
Control	8.88 ± 0.48	1.35 ± 0.12
+ EGF	1.61 ± 0.31	12.29 ± 0.15
+ EGF + EPPA		
15 μ M	0	0
7.5 μ M	0.03 ± 0.01	0.07 ± 0.01
1.0 μ M	0.16 ± 0.03	11.77 ± 1.50
0.5 μ M	0.52 ± 0.16	12.05 ± 1.75
0.1 μ M	1.07 ± 0.32	13.26 ± 1.65
+ EGF + EPA		
15 μ M	0	0
7.5 μ M	0	0.12 ± 0.05
1.0 μ M	0.15 ± 0.03	13.37 ± 0.54
0.5 μ M	0.60 ± 0.20	13.69 ± 0.52
0.1 μ M	1.28 ± 0.07	11.98 ± 0.99

clonogenicity of either MDA-468 or MDA-468-S4 cells. It is interesting to note, however, that both EPA and EPPA seemed to have an additive effect to that of EGF in inducing growth inhibition of MDA-468 in agar, although neither agent, at low concentrations (0.1–1.0 μ M), altered the EGF-dependent clonogenicity of MDA-468-S4. The significance of this observation is obscure, but it may be related to the EGF-receptor gene amplification and EGF-induced growth inhibition characteristic of MDA-468. This effect on MDA-468 seemed to be restricted to anchorage-independent conditions however, as it was absent when the two amiloride analogues were tested under anchorage-dependent conditions (see below and Table 2). At higher concentrations of the amiloride analogues (15 and 7.5 μ M), plating efficiency was significantly depressed regardless of the expected actions of EGF on growth. Experiments performed with MDA-468 cells in anchorage-independent agar culture in the absence of EGF confirmed that non-specific toxic effects of EPA and EPPA were observed at the two higher concentrations (15 and 7.5 μ M). Growth of the cells in amiloride analogue concentrations of 1.0–0.1 μ M was not altered (results not shown).

MDA-468 and MDA-468-S4 cells incorporate [3 H]-thymidine in the absence of stimulation by exogenous growth factors and cannot be rendered quiescent by serum deprivation (Table 2). However, when either cell line is treated with 10^{-8} M-EGF, [3 H]thymidine incorporation is altered in the manner expected from the EGF-mediated alterations in colony formation, i.e. marked decreases in tritium incorporation in MDA-468 cells and increases in MDA-468-S4 cells (Table 2). The concentration of the amiloride analogues which significantly inhibits the Na^+/H^+ exchange specifically (1.0 μ M) was unable to block the EGF-induced effects in

Table 2. Effect of amiloride analogues on EGF-dependent alterations in [3 H]thymidine incorporation

MDA-468 and MDA-468-S4 cells (10^6 /plate) were incubated in HCO_3^- -free medium with EGF (10^{-8} M) and/or amiloride analogues (EPA and EPPA) in the presence of 5 μ Ci of [3 H]thymidine $\cdot \text{ml}^{-1}$ in anchorage-dependent conditions for 6 h as outlined in the Materials and methods section and as shown in the Table. After an 18 h chase in unlabelled, HCO_3^- -free media, counts incorporated were determined by scintillation counting of three replicate plates.

Conditions	[3 H]thymidine uptake (c.p.m.)	
	MDA-468	MDA-468-S4
Control	718680 ± 9512	474088 ± 18469
+ EGF	469698 ± 19561	691285 ± 4830
+ EGF + EPPA		
15 μ M	282825 ± 15586	237435 ± 16503
7.5 μ M	395315 ± 18397	234058 ± 20646
1.0 μ M	470212 ± 13268	568312 ± 29516
0.5 μ M	509210 ± 45815	667274 ± 19331
0.1 μ M	526907 ± 37440	648495 ± 30634
+ EGF + EPA		
15 μ M	288400 ± 14311	315042 ± 20982
7.5 μ M	403955 ± 3342	460190 ± 18379
1.0 μ M	429382 ± 18362	609148 ± 28336
0.5 μ M	510118 ± 21203	614101 ± 28511
0.1 μ M	524285 ± 7935	661311 ± 15922

both cell lines (Table 2). Again, however, at higher concentrations of EPA and EPPA (15 and 7.5 μ M), non-specific inhibition of [3 H]thymidine incorporation was observed (Table 2). These data, therefore, correlate closely with the agar proliferation data described in Table 1.

To examine an earlier manifestation of the cellular responses to mitogens, we examined the increased expression of the nuclear proto-oncogenes *c-myc* and *c-fos* mRNA. Earlier work from this laboratory has shown that EGF can induce transient elevation of the level of transcripts of these two genes in both MDA-468 and MDA-468-S4 cells, even though the proliferative response of the two cell lines to EGF is completely opposite [8, 35]. After 30 min treatment with 10^{-8} M-EGF, the level of *c-myc* and *c-fos* mRNA is increased by 2.2- and 17.5-fold, respectively in MDA-468 cells (Figs. 3a and 3b, lanes 1 and 2). The increase was not significantly altered by 1.0 μ M-EPPA, the analogue and concentration most effective in specifically inhibiting the Na^+/H^+ antiport (Figs. 3a and 3b, lanes 3 and 4). Results from the variant cell line MDA-468-S4 were identical with those reported above for MDA-468 (results not shown).

Effects of preventing the pH_i from rising above the resting pH_i on EGF-dependent mitogenesis

To eliminate the possibility that cytoplasmic alkalization may occur through a mechanism distinct from the Na^+/H^+ antiport or $\text{HCO}_3^-/\text{Cl}^-/\text{Na}^+$ exchange, we examined the response of cells to EGF in media with extracellular pH (pH_o) values from 7.2 to 6.8. In medium with pH_o 6.8, pH_i will not rise above resting values of approx. 7.2 when treated with mitogenic concentrations of growth factors [12]. As shown in Figs. 3(a) and 3(b)

Fig. 3. EGF-Stimulated *c-myc* (a) and *c-fos* (b) gene expression in MDA-468 cells

Cells were incubated in the absence (odd-numbered lanes) or presence (even-numbered lanes) of EGF (10^{-8} M) for 30 min before cell harvest. Other additions in both *a* and *b* are as follows: lanes 1 and 2, no further additions; lanes 3 and 4, *c-myc* and *c-fos* expression in the presence of 1.0 μ M-EPPA; lanes 5 and 6, *c-myc* and *c-fos* expression in medium of pH_o 6.8; and lane 7, *c-myc* and *c-fos* RNA levels induced by 140 mM-*N*-methylglucamine (NMG) alone. In both (a) and (b) poly(A)⁺RNA was examined by Northern-blot analysis with ³²P-labelled nick-translated probes specific for *c-myc* and *c-fos* as outlined in the Materials and methods section. Human 28 S and 18 S ribosomal RNA were used as standards (left).

(lanes 5 and 6), treatment of MDA-468 cells for 30 min with 10^{-8} M-EGF in medium of pH_o 6.8 caused an increase in levels of *c-myc* and *c-fos* mRNA in a similar manner to that occurring in control medium of pH_o 7.2 (Figs. 3a and 3b, lanes 1 and 2). The response of MDA-468-S4 cells at pH_o 6.8 was identical with MDA-468 (results not shown).

We also examined the EGF-dependent alteration in [³H]thymidine incorporation in both MDA-468 and MDA-468-S4 cells in media of decreasing pH_o values. Results for both cell lines in media of four different pH_o are shown in Table 3. At control pH_o 7.2, EGF induced a significant decrease in labelling in MDA-468 and a marked increase in [³H]thymidine counts incorporated in MDA-468-S4, consistent with the data shown in Table 2. At pH_o 6.8, where the pH_i does not increase above resting pH_i, the significant EGF-induced changes in [³H]thymidine incorporation are unaltered.

This suggests that although EGF did induce cytoplasmic alkalization, an increase in pH_i above resting pH_i 7.2 was not obligatory for EGF-dependent growth inhibition or stimulation.

Is activation of the Na⁺/H⁺ antiport sufficient to induce increased *c-myc* and *c-fos* expression?

The exchange of H⁺ for Na⁺, as well as leading to an intracellular alkalization, causes an increase in intracellular Na⁺ concentration ([Na⁺]_i) [9]. It is conceivable that this alone might be necessary or sufficient for increased gene expression. Similarly, it is of interest to examine whether cytoplasmic alkalization itself, through activation of the antiport, might stimulate some degree of mitogenesis, as has been reported in sea urchin eggs [36]. These hypotheses were tested by stimulating the antiport directly with a hyper-osmotic stress with 140 mM-*N*-methylglucamine. A 30 min treatment of MDA-468 and MDA-468-S4 cells with *N*-methylglucamine did not cause significant induction of *c-myc* and *c-fos* mRNA expression in otherwise unstimulated cells. Data illustrating the response in MDA-468 cells are shown in Figs. 3(a) and 3(b) (lanes 7). Results from MDA-468-S4 cells did not differ significantly from those found here (not shown). This indicates that an increase in pH_i by itself, or in combination with an increase in [Na⁺]_i, was incapable of inducing increased levels of either *c-myc* or *c-fos* transcription.

DISCUSSION

A Na⁺/H⁺ antiporter is a constituent of the plasma membrane in virtually all animal cells and can function to extrude protons from the cytoplasm using the inwardly directed Na⁺ gradient. The antiporter activity is dramatically increased by certain stimuli, leading to responses such as volume regulation [37,38], transepithelial ion transport [39], and pH_i regulation [40]. The latter response is known to take place upon activation of the Na⁺/H⁺ exchange by a variety of growth factors [12–14, 41–46] and other bioactive compounds, such as phorbol esters [12, 16, 41]. This activation may be necessary for the emergence of cells from a quiescent state upon the addition of growth factors.

A number of pieces of experimental evidence accumulated over the last decade have been utilized to suggest that activation of the Na⁺/H⁺ antiport and the resultant

Table 3. pH_o dependence of EGF-induced mitogenesis

MDA-468 and MDA-468-S4 cells (10^6 /plate) were incubated in HCO₃⁻-free medium at the indicated pH_o in the presence or absence of EGF (10^{-8} M) in the absence of serum. [³H]Thymidine (5 μ Ci · ml⁻¹) incorporation (c.p.m.) was determined for three replicate wells following a 6 h pulse, followed by an 18 h chase in unlabelled, HCO₃⁻-free medium at the same indicated pH_o. pH_o was determined before and after culture and was not significantly changed at the termination of the experiment.

pH _o	[³ H]Thymidine incorporation (c.p.m.)			
	MDA-468		MDA-468-S4	
	Medium	EGF	Medium	EGF
7.2	206 291 ± 12 564	129 602 ± 6 319	185 039 ± 12 603	251 718 ± 15 928
7.0	145 558 ± 7 482	78 434 ± 2 173	158 483 ± 9 524	232 068 ± 14 982
6.9	131 297 ± 5 858	99 374 ± 4 318	162 283 ± 11 509	229 815 ± 14 816
6.8	137 945 ± 6 847	104 301 ± 5 290	155 056 ± 9 523	228 712 ± 15 119

increase in pH_i may be required for mitogenesis. These include: (1) mutant cells lacking the antiport cannot proliferate in HCO_3^- -free media of low pH_o , and thus low pH_i [47]; (2) growth factor-induced proliferation can only occur over a narrow pH_o range [48], and elevation of pH_o can induce mitogenesis [49]; (3) the concentration of extracellular Na^+ is a limiting factor in growth factor-dependent induction of proliferation [42]; and (4) the K^+ -sparing diuretic, amiloride, and its analogues, which inhibit the Na^+/H^+ antiport, can also block the growth changes due to growth factor stimulation, especially in HCO_3^- -free medium with low extracellular Na^+ concentration [14].

However, the role of Na^+/H^+ exchange and cytoplasmic alkalization in mitogenesis remains an area of considerable scientific controversy. On one hand, findings in sea urchin eggs [36] show that mitogenesis can be induced by simply elevating pH_i . Except for a single report [49], this has not been confirmed in mammalian cells. Similarly, work from Pouyssegur and colleagues [47, 50] have shown conflicting evidence concerning the ability of mutant cells devoid of Na^+/H^+ antiporters to grow at physiological pH_o in the absence of HCO_3^- . Similarly, while concentrations of amiloride shown to be effective in inhibiting Na^+/H^+ exchange also block cell proliferation induced by growth factors, the drug also inhibits a wide range of essential cell growth-dependent reactions [15–18]. Lastly, it is conceivable that in experiments where Na^+/H^+ exchange was precluded and HCO_3^- was absent, pH_i falls below the resting physiological value, reaching levels that are not permissive for the development of the proliferative response.

In contrast, there have been a number of recent publications which do not support the notion that activation of the Na^+/H^+ antiport is required for growth factor-induced proliferation [12, 18, 27, 51, 52]. However, the majority of these studies have been carried out in lymphocytes and/or thymocytes [12, 27, 52] and the possibility exists that these findings are unique to these cell populations.

The purpose of this study was to determine whether activation of Na^+/H^+ exchange is essential to trigger mitogenesis. We have performed these studies in cells other than lymphocytes or thymocytes and in a cellular model that permitted an investigation of the role of changes in pH_i in EGF-induced proliferation and growth inhibition. Furthermore, we have examined the role of the Na^+/H^+ antiporter in cell proliferation over a time course of days (colony formation in semi-solid medium), and in two earlier markers of the proliferative cascade, [^3H]thymidine incorporation and the transient induction of *c-myc* and *c-fos* transcription.

Our data can be summarized as follows. First, at concentrations of EGF that alter the growth of both MDA-468 and MDA-468-S4 cells, we can demonstrate a marked cytoplasmic alkalization in nominally HCO_3^- -free media. This increase in pH_i can be blocked by amiloride and its analogues, EPA and EPPA, and is thus attributable to the Na^+/H^+ antiport (Figs. 1 and 2). Secondly, at concentrations of amiloride analogues EPA and EPPA that nearly completely inhibit the Na^+/H^+ antiport (Fig. 2), few changes in EGF-induced agar growth of MDA-468 or MDA-468-S4 cells could be observed (Table 1). These data are corroborated by the findings that neither amiloride analogue alters EGF-stimulated changes in [^3H]thymidine incorporation in

either cell line at concentrations which specifically inhibit the antiport alone (Table 2).

Previous work from this laboratory has demonstrated that augmented transcription of the nuclear proto-oncogenes *c-myc* and *c-fos* can be transiently induced by concentrations of EGF that result in either growth inhibition in MDA-468 cells or growth stimulation in MDA-468-S4 cells [8, 35]. Effective concentrations of amiloride analogues, EPA and EPPA, were unable to inhibit augmented *c-myc* or *c-fos* transcription in either cell line (Fig. 3).

Although proliferation occurred when Na^+/H^+ exchange and HCO_3^- -dependent processes were prevented, it was possible that an additional, as yet undescribed mechanism for pH_i elevation by EGF could occur. We eliminated this possibility by demonstrating that EGF-induced proliferation, as measured both by induction of *c-myc* and *c-fos* transcription and by [^3H]thymidine uptake, was unaltered from control measurements when performed in nominally HCO_3^- -free medium at pH_o 6.8, where growth factors cannot increase pH_i above the resting pH_i (Fig. 3 and Table 3) [12].

Finally, alterations in intracellular $[\text{Na}^+]$ and $[\text{H}^+]$ as a result of hyper-osmotic stress in otherwise quiescent cells are neither sufficient nor necessary to activate the expression of *c-myc* and *c-fos* mRNA (Fig. 3).

The conclusion obtained from our experiments runs contrary to the notion that stimulation of Na^+/H^+ exchange and cytoplasmic alkalization are necessary for proliferation. It does however agree with some previously published observations [12, 18, 27, 51, 52]. We have extended these by showing that antiport activation is not necessary for mitogenic events to occur in a non-lymphoid human cell line. In addition, we have also ruled out the possibility that, although early stages of the proliferative cascade are independent of alkalization, later stages require pH_i change, by measuring both early and late mitogenic events. Furthermore, we have demonstrated that Na^+/H^+ exchange and increased pH_i occurs in both EGF-dependent growth-stimulated and growth-inhibited cells. While the molecular basis of EGF-dependent growth inhibition remains elusive, we have demonstrated that it is not related to differences in cellular dependence upon an EGF-induced activation of the Na^+/H^+ antiport.

Although the 5-*N*-disubstituted amiloride analogues are more specific inhibitors of the antiport than their parent compound [14, 19], their non-specific effects during extended incubations have not been systematically assessed. Our data demonstrate that non-specific toxicity is associated with two of the analogues, EPA and EPPA, at concentrations greater than $1.0 \mu\text{M}$. This is observed in either long-term proliferation culture or shorter-term [^3H]thymidine uptake studies. Mills *et al.* [27] have demonstrated that these analogues remain active in culture for up to 96 h. Our toxicity studies, the close correlation between data from either early or late steps in the mitogenic process, and the synergistic effect of the analogues with EGF on MDA-468 clonogenicity in agar support this and provide circumstantial evidence that these agents retain their activity well beyond 96 h. The possibility remains to be investigated, however, whether the concentration dependence of the specific and non-specific effects of the agents are unique to the cell lines used in these studies.

In summary, although EGF is shown to activate

Na⁺/H⁺ exchange with concomitant cytoplasmic alkalinization in human breast cancer cells, neither event is an obligatory requirement for EGF-induced mitogenesis, EGF-induced growth inhibition or EGF-induced augmented gene expression.

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REFERENCES

- Carpenter, G. & Cohen, S. (1979) *Annu. Rev. Biochem.* **48**, 193–216
- Cooper, J. A. & Hunter, T. (1983) *Curr. Top. Microbiol. Immunol.* **107**, 125–161
- Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930
- Owen, N. E. & Villereal, M. L. (1983) *J. Cell Physiol.* **117**, 23–29
- Moolenaar, W. H., Tertoolen, L. G. J. & de Laat, S. W. (1984) *J. Biol. Chem.* **259**, 8066–8069
- Kelly, K., Cochran, B. H., Stiles, C. D. & Leder, P. (1983) *Cell (Cambridge, Mass.)* **35**, 603–610
- Muller, R., Bravo, R., Buckhead, J. & Curran, T. (1984) *Nature (London)* **312**, 716–720
- Filmus, J., Benchimol, S. & Buick, R. N. (1987) *Exp. Cell Res.* **169**, 554–559
- Seifter, J. L. & Aronson, P. S. (1986) *J. Clin. Invest.* **78**, 859–864
- Grinstein, S. & Rothstein, A. (1986) *J. Membr. Biol.* **90**, 1–12
- Moolenaar, W. H. (1986) *Annu. Rev. Physiol.* **48**, 363–376
- Mills, G. B., Cragoe, E. J., Jr., Gelfand, E. W. & Grinstein, S. (1985) *J. Biol. Chem.* **260**, 12500–12507
- Moolenaar, W. H., Yarden, Y., de Laat, S. W. & Schlessinger, J. (1982) *J. Biol. Chem.* **257**, 8502–8506
- L'Allemain, G., Franchi, A., Cragoe, E. J., Jr. & Pouyssegur, J. (1984) *J. Biol. Chem.* **259**, 4313–4319
- Davis, R. J. & Czech, M. P. (1985) *J. Biol. Chem.* **260**, 2543–2551
- Besterman, J. M., May, W. S., Jr., LeVine, H., III, Cragoe, E. J., Jr. & Cuatrecasas, P. (1985) *J. Biol. Chem.* **260**, 1155–1159
- Koch, K. S. & Leffert, H. L. (1979) *Cell (Cambridge, Mass.)* **18**, 153–163
- Besterman, J. M., Tyrey, S. J., Cragoe, E. J., Jr. & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6762–6766
- Cragoe, E. J., Jr., Woltersdorf, O. W., Jr., Bicking, J. B., Kwong, S. F. & Jones, J. H. (1967) *J. Med. Chem.* **10**, 66–75
- Filmus, J., Pollack, M. N., Cailleau, R. & Buick, R. N. (1985) *Biochem. Biophys. Res. Commun.* **128**, 898–905
- Filmus, J., Trent, J. M., Pollack, M. N. & Buick, R. N. (1987) *Mol. Cell. Biol.* **7**, 251–257
- Gill, G. N. & Lazar, C. S. (1981) *Nature (London)* **293**, 305–307
- Pathak, S., Siciliano, M. J., Cailleau, R., Wiseman, C. L. & Hsu, T. L. (1979) *J. Natl. Cancer Inst.* **62**, 263–271
- Jones-Grucigar, Q. V., Pathak, S. & Cailleau, R. (1976) *Cytogenet. Cell Genet.* **17**, 231–235
- Grinstein, S., Cohen, S., Goetz, J. D., Rothstein, A. & Gelfand, E. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1429–1433
- Thomas, J. A., Buchsbaum, R. N., Zimniak, E. & Racker, E. (1979) *Biochemistry* **18**, 2210–2218
- Mills, G. B., Cheung, R. K., Cragoe, E. J., Jr., Grinstein, J. & Gelfand, E. W. (1986) *J. Immunol.* **136**, 1150–1154
- Grinstein, S., Cohen, S., Goetz, J. D. & Rothstein, A. (1985) *J. Cell. Biol.* **101**, 269–276
- Chirgwin, J. M. (1979) *Biochemistry* **8**, 5294–5299
- Dalla-Favera, R., Wong-Staal, F. & Gallo, R. C. (1982) *Nature (London)* **299**, 61–63
- Curran, T., Peters, G., Van Beveren, C., Teich, N. M. & Verma, I. M. (1982) *J. Virol.* **44**, 674–682
- Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251
- Jentsch, T. J., Stahlknecht, T. R., Hollwede, H., Fisher, D. G., Keller, S. K. & Wiedesholt, M. (1985) *J. Biol. Chem.* **260**, 795–801
- Roos, A. & Boron, W. F. (1981) *Physiol. Rev.* **61**, 296–434
- Church, J. G. & Buick, R. N. (1988) *J. Biol. Chem.* **263**, 4242–4246
- Johnson, J. J., Epel, D. & Paul, M. (1976) *Nature (London)* **262**, 661–664
- Cala, P. M. (1980) *J. Gen. Physiol.* **76**, 683–708
- Grinstein, S., Cohen, S., Goetz, J. D. & Rothstein, A. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 2508–2512
- Aronson, P. S. (1983) *Am. J. Physiol.* **245**, F647–F659
- Madhus, I. H. (1988) *Biochem. J.* **250**, 1–8
- Hesketh, T. R., Moore, J. P., Morris, J. D. H., Taylor, M. V., Roger, J., Smith, G. A. & Metcalfe, J. C. (1985) *Nature (London)* **313**, 481–484
- Burns, C. P. & Rozengurt, E. (1983) *Biochem. Biophys. Res. Commun.* **116**, 931–938
- Paris, S. & Pouyssegur, J. (1986) *EMBO J.* **5**, 55–60
- Huang, C.-L., Cogan, M. G., Cragoe, E. J., Jr. & Ives, H. E. (1987) *J. Biol. Chem.* **262**, 14134–14140
- Rothenberg, P., Glaser, L., Schlesinger, P. & Cassel, D. (1983) *J. Biol. Chem.* **258**, 12644–12653
- Vara, F. & Rozengurt, E. (1985) *Biochem. Biophys. Res. Commun.* **130**, 646–653
- Pouyssegur, J., Sardet, C., Franchi, A., L'Allemain, G. & Paris, S. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4833–4837
- Taylor, I. W. & Hodson, P. J. (1984) *J. Cell Physiol.* **121**, 517–525
- Zetterberg, A. & Engstrom, W. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4334–4338
- Pouyssegur, J., Franchi, A., L'Allemain, G. & Paris, S. (1985) *FEBS Lett.* **190**, 115–119
- Novak-Hofer, I., Kung, W. & Eppenberger, U. (1988) *J. Cell Biol.* **106**, 395–401
- Grinstein, S., Smith, J. D., Onizuka, R., Cheung, R. K., Gelfand, E. G. & Benedict, S. (1988) *J. Biol. Chem.* **263**, 8658–8665