Epidermal Growth Factor Stimulates Amiloride-sensitive $^{22}\text{Na}^+$ Uptake in A431 Cells

EVIDENCE FOR Na$^+$/H$^+$ EXCHANGE*

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Epidermal growth factor (EGF) increases Na$^+$ uptake in several cell types through an electroneutral, amiloride-sensitive pathway putatively identified as Na$^+$/H$^+$ countertransport. The inferred cytosolic alkalization resulting from this process has been proposed to be an important component of mitogenic stimulation. We studied the effect of EGF on the Na$^+$/H$^+$ exchange system of A431 cells, a cell line having a very high EGF receptor density but which is not mitogenically stimulated by EGF. We demonstrate that EGF rapidly activates net Na$^+$ influx in A431 cells. Amiloride inhibits the EGF-dependent Na$^+$ uptake (65% inhibition at 3 mM, $I_{50} \sim 0.3$ mM) and inhibits much less the EGF-independent uptake. EGF is known to enhance $^{40}\text{Ca}^+$ accumulation in A431 cells (Sawyer, S. T., and Cohen, S. (1981) Biochemistry 20, 6280-6286). The following findings indicate that EGF-dependent $^{22}\text{Na}^+$ and $^{40}\text{Ca}^+$ uptake are two independent processes. 1) EGF effectively stimulates an amiloride-sensitive $^{22}\text{Na}^+$ uptake in the absence of external Ca$^{2+}$. 2) EGF-dependent $^{40}\text{Ca}^+$ uptake is not inhibited by amiloride.

A new fluorescence technique is described for intracellular pH determination based on the introduction of fluorescein-labeled dextran into the cell cytoplasm. Using this method, the presence of amiloride-sensitive Na$^+$/H$^+$ exchange in A431 cells is documented. Although the lack of pH sensitivity of fluorescein fluorescence above pH 7.3-7.4 prevents a direct assessment of an EGF-induced increase of intracellular pH, the combined results of $^{22}\text{Na}^+$ flux and intracellular pH measurements suggest that EGF activates Na$^+$/H$^+$ exchange in A431 cells. We conclude that enhanced Na$^+$/H$^+$ exchange may not necessarily be coupled to mitogenic triggering in different cell types, although the stimulation of Na$^+$/H$^+$ exchange may constitute a primary event in the mechanism of EGF action.

Epidermal growth factor, a mitogenic polypeptide discovered by Cohen (for review, see Ref. 1) binds to specific cell surface receptors, triggering multiple biochemical and physiological responses. EGF rapidly activates such processes as increased tyrosine-specific protein kinase activity (2, 3), augmented nutrient transport (4, 5), cytoskeletal rearrangements (6, 7), receptor internalization (8, 9), and alterations in transmembrane ion fluxes (10, 11). A defined scheme for the mechanism of EGF action has not yet emerged, and it is unclear which, if any, of such early cellular responses are key components of EGF-induced cell proliferation.

Na$^+$ influx quickly increases in cells mitogenically stimulated by serum or defined growth factors, including EGF (12). Because this Na$^+$ entry is electroneutral as demonstrated by electrophysiological measurements, and is sensitive to the diuretic amiloride, it may occur through a stoichiometric Na$^+$/H$^+$ countertransport mechanism (11, 13). Na$^+$/H$^+$ exchange is involved in the regulation of intracellular pH (for review, see Ref. 14), and growth factor-mediated activation of Na$^+$/H$^+$ exchange may elevate cytosolic pH. The manifold effects on cellular metabolism and organization that may result from such an alkaline shift of intracellular pH have been suggested to play a central role in the initiation of cell growth and division (15, 16).

The human epidermoid carcinoma cell line, A431, bears an exceptionally high surface density of EGF receptors (17, 18), and this feature has facilitated studies of EGF action by amplifying some of the effects of EGF also observed in normal, nonmalignant cells. Despite the stimulation by EGF of early events such as tyrosine phosphorylation (2, 3) in A431 cells, EGF is not mitogenic for these cells and can inhibit cell growth after prolonged incubation (19, 20). If intracellular alkalization occurring through Na$^+$/H$^+$ exchange is obligatory for the entry of quiescent cells into the cell cycle, then it might be presumed that EGF would not stimulate Na$^+$/H$^+$ exchange in an already continuously growing, transformed cell line such as A431. However, if activated Na$^+$/H$^+$ exchange is a primary component of the mechanism of EGF action, then such exchange fluxes should also be observed in A431 cells, despite the lack of mitogenic response to EGF. In this report, we demonstrate that EGF enhances $^{22}\text{Na}^+$ uptake in A431 cells, apparently through a Na$^+$/H$^+$ exchange mechanism. These results suggest that the activation of Na$^+$/H$^+$ exchange is not necessarily coupled to the induction of cellular proliferation by EGF.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: $^{22}\text{Na}^+$, $^{40}\text{Ca}^+$ (New England Nuclear); dextran, fluorescein isothiocyanate, Dulbecco’s modified Eagle’s medium; Hepes, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; TMA-Cl, tetramethylammonium chloride; pH, intracellular pH; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N’,N’’,N’’-tetraacetic acid.

$^{1}$ The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco’s modified Eagle’s medium; Hepes, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; TMA-Cl, tetramethylammonium chloride; pH, intracellular pH; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N’,N’’,N’’-tetraacetic acid.

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anate, insulin, and ouabain (Sigma); polyethylene glycol 1500 (500-600 average molecular weight) (Fisher); bovine serum (K. C. Biologicals, Inc.). Amiloride was generously provided by A. W. Alberts of Merck Sharp and Dohme. Platelet-derived growth factor-depleted bovine fetal calf plasma was prepared as described (21). EGF was prepared by the method of Savage and Cohen (29). Purified platelet-derived growth factor was a gift of J. H. Huang and T. Deuel, Washington University. Fluorescein-dextran was synthesized and purified essentially as described by DeBelder and Granath (23). All other materials were obtained from commercial sources and were of the highest purity available.

**Cell Culture**—The A431 cell line, obtained from Dr. C. F. Fox, University of California, Los Angeles, was maintained in DME containing 100 units/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 7% newborn calf serum and 3% fetal calf serum, in Falcon tissue culture plastic ware at 37° C. Cell numbers were determined after trypsinization in a Coulter counter.

**Na**<sup>+</sup> Uptake Measurements—A431 cells were plated in 35-mm dishes at densities between \(2.2 \times 10^5\) and \(2.5 \times 10^5\) cells/cm<sup>2</sup>, and grown to near confluency in DME with 10% serum. About 12-18 h prior to use, the cultures were shifted to DME without NaHCO<sub>3</sub> and NaCl was replaced by an equimolar amount of TMA-C1 or N-methyl-d-glucamine chloride. **Na**<sup>+</sup> uptake was initiated by addition of 0.9 ml of saline solution containing **Na**<sup>+</sup> (1.6-2.2 \(\times\) 10<sup>4</sup> cpm) and ouabain (0.5 or 1.0 mm), with or without 200 ng of EGF. Where indicated, amiloride was added to saline solutions from a 1 mM stock solution in dimethyl sulfoxide; equivalent dimethyl sulfoxide concentrations less than 0.4% (final volume) were added to controls. At these concentrations, dimethyl sulfoxide was found to be without effect upon **Na**<sup>+</sup> uptake. Following incubation at 37° C, **Na**<sup>+</sup> uptake was terminated by aspirating the medium and rapidly washing by sequentially dipping the dish in 4 beakers containing 500 ml of cold 0.1 M NaCl, 10 mM Tris, pH 7.5. Cells were extracted at room temperature with 2 ml of 5% trichloroacetic acid, and the radioactivity in the extract determined in a Beckman gamma counter. All assays were done in triplicate, and standard deviations were regularly less than 10% of the mean.

**Intracellular pH Measurements**—Cells were grown to confluency on small glass slides (0.9 \(\times\) 2.5 cm) resting in 35-mm dishes. The "osmotic lysis" technique recently developed by Okada and Rechsteiner (24) was used to introduce fluorescein-labeled dextran (average molecular weight, 10,000; about 0.3 fluorescein group:dextran molecule) into the cell cytoplasm. Briefly, cells were allowed to pinocytose for 10 min at 37° C in a hypertonic solution (DME-Hepes with 0.5 M sucrose and 10% polyethylene glycol) containing 250 mg/ml of fluorescein-dextran. Cells were then washed with three, 3-ml aliquots of warm DME made hypotonic by dilution with water (6 parts H<sub>2</sub>O:4 parts DME), and incubated in this hypotonic DME for 2 min at 37° C. The hypotonic solution was then replaced with 2 ml of DME-Hepes, 1% fetal calf serum, and the cells were incubated overnight before use. The distribution of cellular fluorescence was examined with a Zeiss epi-fluorescence microscope. Commercial preparations of fluorescein-dextran (Sigma) were found to be cytotoxic, and some lots of polyethylene glycol were also toxic, perhaps due to peroxide formation.

Intracellular pH was determined by measuring the fluorescence emitted from the monolayer at 516 nm upon excitation at 495 and 450 nm in an Amino SPF-600 spectrofluorometer. The glass slide supporting the cell monolayer was mounted at an angle of 45° relative to the excitation beam in a holder similar to that described by Ohkuma and Poole (25). The cuvette temperature was maintained at 37° C. The fluorescence spectrum of fluorescein is sensitive to pH, and the ratio of fluorescence intensities at two wavelengths provides an index of pH which is independent of dye concentration and optical path-length. A more complete discussion of this principle has been presented elsewhere (26).

**RESULTS**

**EGF-dependent **Na**<sup>+</sup> Uptake—Confluent monolayers (1.0-1.5 \(\times\) 10<sup>5</sup> cells/dish) of A431 cells in 35-mm dishes were incubated at 37° C for about 18 h in nominally HCO<sub>3</sub>−-free DME containing 25 mM Hepes, pH 7.4, supplemented with 1% (v/v) fetal calf serum. Cells were then incubated at 37° C in a saline solution containing **Na**<sup>+</sup> and ouabain, with or without EGF. As shown in Fig. 1, addition of EGF stimulated **Na**<sup>+</sup> uptake by the cells. The EGF-dependent uptake is essentially linear for the first 3 min of incubation and increases more slowly thereafter. The basal uptake levels off at early time points, and shows a second increase after 30-40 min; this second increase was consistently observed in several experiments. A possible explanation for this deviation from simple exponential kinetics is presented later. Both in the presence and absence of 200 ng/ml of EGF, **Na**<sup>+</sup> uptake attained the same final value after incubation for 2 h (Fig. 1) indicating that EGF does not alter the capacity of the cells for **Na**<sup>+</sup>. EGF also failed to alter significantly the amount of **Na**<sup>+</sup> accumulated by monolayers incubated in saline solution for 2 and 3 h in the absence of ouabain (not shown).

**Fig. 2** shows that the EGF-dependent **Na**<sup>+</sup> uptake rate is half-maximal at about 10 ng/ml, and approaches saturation at higher EGF concentrations. While the concentration dependence of EGF binding to its surface receptors on the cells under our experimental conditions has not been investigated, the observed sensitivity of **Na**<sup>+</sup> uptake of EGF is similar to that reported for other actions of EGF on A431 cells, such as the induction of membrane ruffling (6, 7), calcium uptake and phosphatidylinositol turnover (27), and tyrosine phosphorylation.

**Fig. 1. Time course of basal and EGF-stimulated **Na**<sup>+</sup> uptake by A431 cells.** Uptake was carried out at 37° C in saline solution (125 mM **Na**<sup>+</sup>) containing **Na**<sup>+</sup> and ouabain. EGF was added at 200 ng/ml (○) at zero time. Basal uptake is given as open circles (○), also plotted is the difference between the two conditions (△). All points represent the mean of triplicate samples and are accurate to within 5%.
ulation reactions (28). Villereal (10) has previously demonstrated EGF-enhanced \(^{22}\text{Na}\) influx into serum-deprived human fibroblasts, with half-maximal effect at 7.5 ng/ml.

We tested the effect of the diuretic drug, amiloride, on EGF-stimulated \(^{22}\text{Na}\) uptake to assess whether the hormone-enhanced influx was mediated by an Na\(^+\)/H\(^+\) exchange mechanism. Fig. 3 shows that amiloride inhibits EGF-dependent \(^{22}\text{Na}\) uptake while inhibiting basal \(^{22}\text{Na}\) uptake only slightly. The amiloride concentration yielding half-maximal inhibition of EGF-dependent \(^{22}\text{Na}\) uptake (~0.3 mM) is comparable to concentrations of the drug inhibiting Na\(^+\)/H\(^+\) exchange in other cell systems in the presence of physiological external Na\(^+\) levels (for review, see Ref. 29). Inhibition of the EGF-dependent uptake did not exceed 60-65%, even at the highest drug concentrations employed. Preincubating the cells with amiloride for 10 min prior to addition of \(^{22}\text{Na}\) and EGF only marginally improved the degree of inhibition (results not shown). The relatively high concentrations of amiloride required to inhibit the EGF-dependent \(^{22}\text{Na}\) influx in A431 cells contrasts with the low (micromolar) levels of this diuretic known to inhibit electrodiffusional Na\(^+\) entry in several epithelia (29). Amiloride does not affect the binding of EGF to its receptor on human fibroblasts (11).

As found for other cell types, serum stimulates \(^{22}\text{Na}\) uptake in A431 cells (Fig. 4). Addition of 10% newborn calf serum increases \(^{22}\text{Na}\) uptake in 10 min to an extent somewhat greater than that produced by maximal doses of EGF. The serum stimulation is amiloride-sensitive. Platelet-derived growth factor contained in serum is not likely to be responsible for the serum effect because: 1), platelet-derived growth factor-depleted plasma was nearly as effective as newborn calf serum (Fig. 4); and 2), pure platelet-derived growth factor (1 µg/ml) did not increase \(^{22}\text{Na}\) uptake under these conditions (not shown). The effects of newborn calf serum and EGF on \(^{22}\text{Na}\) uptake were partially additive. Since newborn calf serum and EGF were each added at maximally stimulatory concentrations, this latter result indicates that EGF and serum act independently, and that the stimulation of \(^{22}\text{Na}\) uptake by serum alone is not due to the presence of EGF in the serum. Bovine insulin (10 µg/ml) did not affect \(^{22}\text{Na}\) uptake (not shown). The factor(s) in serum which stimulates \(^{22}\text{Na}\) uptake into A431 cells remains unidentified. Serum- or EGF-enhanced bulk phase fluid pinocytosis in A431 cells (7) contributes insignificantly to the isotope accumulations reported here.

**Relation of EGF-dependent Na\(^+\) and Ca\(^{2+}\) Uptake**—Sawyer and Cohen (27) have demonstrated EGF-stimulated \(^{45}\text{Ca}\) uptake in A431 cells, and Owen and Villereal (32) have reported inhibition of stimulation, amiloride-sensitive \(^{22}\text{Na}\) uptake into quiescent fibroblasts by phenothiazine calmodulin antagonists. It was therefore of interest to investigate the role of external Ca\(^{2+}\) in EGF-dependent \(^{22}\text{Na}\) uptake. Fig. 5 compares \(^{22}\text{Na}\) uptake in saline containing a normal Ca\(^{2+}\) concentration of 1.8 mM and in Ca\(^{2+}\)-free saline solution containing 0.2 mM EGTA. In the presence of EGTA, EGF still augmented \(^{22}\text{Na}\) uptake in an amiloride-sensitive manner (Fig. 5A). Both basal and EGF-dependent \(^{22}\text{Na}\) uptake were increased in the absence of Ca\(^{2+}\) relative to controls in normal saline (Fig. 5B). External Ca\(^{2+}\) is apparently not required for EGF-dependent \(^{22}\text{Na}\) uptake. As Table I indicates, EGF-dependent \(^{45}\text{Ca}\) uptake in A431 cells is not inhibited by amiloride. These results suggest the presence of two independent, EGF-stimulated ion transport systems in A431 cells. The mechanism by which the interaction of EGF

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**Table I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cpm ± S.D. (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2133 ± 77</td>
</tr>
<tr>
<td>1 mM amiloride</td>
<td>2078 ± 87</td>
</tr>
<tr>
<td>EGF</td>
<td>3585 ± 150</td>
</tr>
<tr>
<td>EGF + 1 mM amiloride</td>
<td>3575 ± 39</td>
</tr>
</tbody>
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**Fig. 3. Effect of amiloride on \(^{22}\text{Na}\) uptake.** Uptake was assayed for 10 min in the presence of the indicated amiloride concentrations with (●) or without (○) EGF (200 ng/ml).

**Fig. 4. Effect of serum on \(^{22}\text{Na}\) uptake.** Uptake was assayed for 10 min. Amiloride was added to give 2 mM final concentration. Bovine sera were dialyzed against saline solution in 3500 molecular weight cut-off tubing before use. Error bars, standard deviation of triplicate determinations. In the right-most pair of bars, the concentrations of EGF and NCS (new born calf serum) were 300 ng/ml and 10% (v/v), respectively.

**Fig. 5. Effect of external [Ca\(^{2+}\)] on \(^{35}\text{Na}\) uptake.** Cells were washed once with saline solution (B) or in saline without CaCl\(_2\) and with 0.2 mM EGTA (A), and incubated for 6 min in the corresponding solution containing \(^{35}\text{Na}\) and the indicated additions. Hatched bars, the presence of 1 mM amiloride. Error bars, standard deviations of triplicate determinations.
with its surface receptor activates these two ion fluxes remains to be established.

**Mechanism of EGF-dependent \(^{22}\text{Na}^+\) Uptake**—In the presence of ouabain, increased \(^{22}\text{Na}^+\) uptake at long time points could reflect a net \(
\text{Na}^+\) influx due to either an increased unidirectional influx or a decreased unidirectional efflux. Additionally, enhanced transmembrane \(
\text{Na}^+\)/\(\text{Na}^+\) exchange could account for some fraction of the observed stimulation of \(^{22}\text{Na}^+\) uptake because the specific activity of the intracellular \(\text{Na}^+\) pool is initially less than the specific activity of the extracellular \(\text{Na}^+\) pool. This effect would therefore be limited by the magnitude of the initial (pre-ouabain) intracellular \(\text{Na}^+\) pool. The intracellular \(\text{Na}^+\) compartment was estimated by equilibrating monolayers in normal saline without ouabain and with \(^{22}\text{Na}^+\) for 2 and 3 h. The size of the \(\text{Na}^+\) pool was 25 ± 1.2 nmols/10^6 cells (mean ± S.D.; \(n = 4\)). Therefore, the contribution of \(\text{Na}^+\)/\(\text{Na}^+\) exchange cannot account for more than about 50% of the EGF-dependent \(\text{Na}^+\) entry during the first 10 min of assay (see Fig. 1).

The effect of EGF on the rate of \(^{22}\text{Na}^+\) efflux was determined by preloading cells with \(^{22}\text{Na}^+\) in the presence of ouabain for 20 min, and subsequently measuring the rate of loss of cell-associated radioactivity after switching the cells to \(^{22}\text{Na}^+\)-free saline with and without EGF. A semi-logarithmic plot of efflux kinetics is shown in Fig. 6. There was no effect of EGF on the rate of \(^{22}\text{Na}^+\) exit from the cells. This result indicates that the observed stimulation of \(^{22}\text{Na}^+\) uptake by EGF is indeed a net inward \(\text{Na}^+\) flux caused by an increased entry rate. Moreover, if \(\text{Na}^+\)/\(\text{Na}^+\) exchange were facilitated by EGF as discussed previously, then the rate of \(^{22}\text{Na}^+\) efflux would also be increased by EGF. The lack of effect of EGF on \(^{22}\text{Na}^+\) efflux therefore indicates that \(\text{Na}^+\)/\(\text{Na}^+\) exchange does not significantly contribute to the observed stimulation of uptake.

We also assessed the effect of EGF on \(^{22}\text{Na}^+\) efflux from cells preloaded with \(^{22}\text{Na}^+\) by preincubation with ouabain for 2 h, rather than 20 min. In these experiments, the loss of \(^{22}\text{Na}^+\) again followed first order kinetics, and the rate constant was unaltered by EGF (results not shown). However, EGF also failed to augment \(^{22}\text{Na}^+\) uptake after exposure to ouabain for 1 h or longer (Fig. 7). Therefore, results obtained from efflux experiments conducted after prolonged ouabain exposure cannot be used to draw conclusions concerning the mechanism of EGF-dependent \(^{22}\text{Na}^+\) uptake. The lack of EGF influence on \(^{22}\text{Na}^+\) entry after prolonged exposure to ouabain is probably due to increased basal \(\text{Na}^+\)/\(\text{H}^+\) exchange activity. As Fig. 7 shows, with preincubation time in ouabain-containing saline, amiloride-sensitive \(^{22}\text{Na}^+\) uptake constitutes a progressively increasing fraction of the basal \(^{22}\text{Na}^+\) uptake. The apparent activation of amiloride-sensitive \(\text{Na}^+\)/\(\text{H}^+\) exchange by prolonged exposure to ouabain (in the absence of EGF) can also be demonstrated by intracellular pH measurements, as will be described later. The augmentation of amiloride-sensitive \(\text{Na}^+\) influx with time in ouabain-containing saline described here may be related to the increased rate of basal \(^{22}\text{Na}^+\) influx after 30-40 min previously noted in Fig. 1. Although the basis for the activation of \(\text{Na}^+\)/\(\text{H}^+\) exchange by preincubation with ouabain is unclear, it may represent some aspect of normal cellular homeostatic mechanisms responding to perturbations caused by inhibition of the (Na,K)ATPase, such as alterations of cell volume, pH, and other intracellular ionic activities.

**Intracellular pH Measurements**—Attempting to determine if EGF-dependent \(^{22}\text{Na}^+\) uptake is mediated through a \(\text{Na}^+\)/\(\text{H}^+\) exchange process which alkalizes the cytosol, we employed a new technique for measuring intracellular pH. Fluorescein-labeled dextran was introduced into the cytosol by a slightly modified version of the "osmotic lysis" procedure developed by Okada and Rechsteiner (24) and served as a sensitive fluorometric indicator of intracellular pH. For at least one day after this loading procedure, the fluorescein-dextran remained diffusely distributed in the cytoplasm and the cells remained fully viable as assessed by trypan blue exclusion or by measurement of basal and EGF-stimulated \(^{22}\text{Na}^+\) uptake. The fluorescence spectrum of fluorescein is pH-sensitive, and a plot of the ratio of fluorescence intensity measured at excitation wavelengths of 495 and 450 nm and an emission wavelength of 516 nm versus pH is shown in Fig. 8. In principle, the use of a ratio measurement permits the accurate determination of pH without knowledge of dye concentration or optical path length. Also shown in Fig. 8 is an in situ calibration of the pH sensitivity of fluorescein-labeled dextran in A431 cells obtained by the nigericin-high K⁺ technique described by Thomas et al. (31). The differences between the two curves shown in Fig. 8 are attributed to the combined effects of light scattering, concentration-dependent quenching of fluorescence, and alteration of the pKₐ of covalently-linked fluorescein. Using this method, the pH of fluorescein-dextran "loaded" but otherwise untreated A431 cells was estimated to be at least 7.3. Because the fluorescence ratio is nearly maximal at this pH (Fig. 8), this method cannot
be used to detect increases of \( pHi \) above the normal \( pHi \) in A431 cells. This limitation has prevented a direct assessment of the possible alkalinizing effects of EGF on cytosolic pH. However, the method is adequate to permit a qualitative demonstration of an amiloride-sensitive \( Na^+ \)/\( H^+ \) mechanism in A431 cells, as shown in Figs. 9 and 10.

For the experiment depicted in Fig. 9, a fluorescein-dextran "loaded" cell monolayer was incubated in a normal saline (125 mM NaCl) solution, containing 1 mM ouabain, for 2 h prior to initiating the fluorescence measurements. After this incubation period, it can be reasonably assumed that intracellular \( [Na^+] \) is also 125 mM. The fluorescence ratio (F 495/450) decreased in a stepwise manner as the \( Na^+ \) concentration in the solution bathing the cells was sequentially halved by exchanging the solution for an otherwise identical one in which NaCl was partly replaced by TMA-Cl. As the ratio of \( [Na^+]_{os}/[Na^+]_{ci} \) decreased, at a constant external \( pHi \), the ratio of \( [H^+]_{os}/[H^+]_{ci} \) increased, causing the observed decrease in cytosolic \( pHi \). Sequential buffer exchanges returning the \( [Na^+] \) to its initial level of 125 mM were accompanied by incremental increases of the fluorescence ratio as the \( pHi \) also returned to its initial level, indicating the reversibility of the process. Note that the observed changes in the fluorescence ratio are not linearly related to \( pHi \) changes, because of the sigmoid nature of the \( pHi \) sensitivity of fluorescein fluorescence (Fig. 8). These observations are consistent with the presence of transmembrane \( Na^+ \)/\( H^+ \) exchange as a consequence of which cytosolic \( pHi \) is in part determined by the \( Na^+ \) concentration gradient across the plasma membrane.

At \( t = 41 \) min in Fig. 9, the normal saline solution was replaced with a 16 mM NaCl, 109 mM TMA-Cl saline solution. The apparent \( pHi \) decreases initially but then smoothly increases back towards its initial level over the next 45 min. This observation reflects the passive \( Na^+ \) permeability of the cell membrane: as \( Na^+ \) slowly leaves the cytoplasm, the intracellular \( Na^+ \) activity approaches the extracellular \( Na^+ \) activity, and the ratio of \( [Na^+]_{os}/[Na^+]_{ci} \) approaches unity. Consequently, \( pHi \) approaches \( pHi \). Upon returning the extracellular \( Na^+ \) concentration back to 125 mM (at \( t = 93 \) min), the \( pHi \) is observed to increase back to about its original level. Because intracellular \( Na^+ \) had been reduced to nearly 15 mM by the preceding incubation in 16 mM Na, 109 mM TMA-Cl (between 0.8 and 0.9 min) abruptly increasing \( [Na^+]_{es} \) to 125 mM (at \( t = 93 \) min) should have imposed a \( Na^+ \) concentration gradient across the cell membrane which would cause the extrusion of protons from the cytoplasm. Heme, a marked intracellular alkalinization to a \( pHi \), exceeding the initial \( pHi \), was anticipated. No such alkalinization was observed. This anomaly results from the restricted \( pHi \) sensitivity of fluorescein fluorescence. As seen in Fig. 8, the fluorescence ratio does not increase above a \( pHi \) of about 7.3.

At \( t = 98 \) min in Fig. 9, the normal saline was again exchanged for 16 mM Na, 109 mM TMA-Cl saline. The \( pHi \), again dropped, consistent with a \( Na^+ \) efflux-driven \( H^+ \) influx. Intracellular acidification was now less extensive than had been previously observed because the prolonged incubation in low Na saline (between 41 and 93 min) had reduced the intracellular \( [Na^+] \) and the gradient then favoring \( Na^+ \) efflux upon reduction of extracellular \( [Na^+] \) was now diminished.

Amiloride inhibits the \( Na^+ \)/\( H^+ \) exchange mechanism in A431 cells. In Fig. 10, cells which had been preincubated for 2 h in normal (125 mM NaCl) saline with 1 mM ouabain were exposed to 1 mM NaCl saline in the presence or absence of 1.2 mM amiloride. Amiloride completely blocked the intracellular acidification caused by reduction of extracellular \( Na^+ \). Upon removal of the amiloride, \( pHi \), decreased normally in low Na+...
saline. At t = 30 min in Fig. 10, the 1 mM Na+ saline was replaced by normal (125 mM Na+) saline containing 1.2 mM amiloride. The rate of increase of pH, caused by enhanced Na+/H+ exchange across the plasma membrane, and to investigate the relationship of Na+/H+ exchange to EGF action. Evidence from a number of laboratories, as well as our own, documents rapidly increased Na+ entry into many quiescent cell types by a variety of mitogens. Electroneutrality of the Na+ entry is an essential criterion for stoichiometric Na+/H+ countertransport, and in both EGF-stimulated human fibroblasts (11), and serum-stimu-
lated monkey epithelial cells (13), direct electrophysiological recording of membrane voltage with intracellular microelec-
trodes has demonstrated the electrically silent nature of the Na+ influx. Because alternate modes of electroneutral Na+ transport are known, such as the Na+/K+/2Cl- co-transport system (33), the criterion of electroneutrality is necessary, but insufficient, evidence for Na+/H+ exchange. The diuretic amiloride inhibits Na+/H+ exchange in muscle (34), epithelia (35), and invertebrate oocytes (15) at millimolar concentra-
tions. The inhibitory effect of amiloride at mM levels on mitogen-stimulated Na+ influx (10, 11, 36) is therefore con-
sistent with an interpretation of this flux as being mediated through Na+/H+ exchange. However, activation of Na+/H+ exchange may not be associated with an alteration of pH, if such activation is secondary to augmented metabolic acid production or if other, as yet unidentified, compensatory ion fluxes are involved. Definitive proof of growth factor induced cytosolic alkalinization requires direct measurements of intra-
cellular pH.

Two main conclusions are supported by the results pre-
sented in this paper. First, EGF initiates net Na+ entry into A431 cells through a pathway which is sensitive to amiloride at millimolar concentrations. Second, A431 cells bear an Na+/H+ exchange mechanism which is also inhibited by amiloride. The isotope tracer experiments required the use of ouabain. Because ionic and probably metabolic perturbations of cell function result from inhibition of the (Na,K)ATPase, conclusions concerning the effects EGF may produce in intact cells (not ouabain-treated) must be cautiously inferred. With this caveat, our results strongly suggest that EGF activates Na+/H+ exchange in A431 cells. Recent studies have shown that EGF actually inhibits the growth of A431 cells (19, 20). This antiproliferative effect places our results in seeming con-
tradiction to the general notion of mitogenic stimulation through Na+/H+ exchange-mediated intracellular alkalinization. We cannot as yet provide a fully satisfactory explanation for this matter, though we speculate that an excess alkalinization caused by EGF in A431 cells, beyond an optimal pH, may restrict cell growth. Alternatively, hormone-induced pH, elevation may elicit varying responses in different cell types, in a manner analogous to the known diversity of second messenger

functions served by cAMP. Activation of Na+/H+ exchange may be an epiphenomenon merely associated with, but not required for, the onset of cell proliferation. However, judgement on this issue should be postponed until adequate measurements of intracellular pH are secured.

The fluorescein-dextran fluorescence ratio method for in-
tracellular pH determination described in this study provides a potentially suitable method with which to investigate these questions. We initially employed the method described by Thomas et al. (31) to measure pH. This method relies on the intracellular hydrolysis of membrane-permeant 6-carboxylflu-
orescein diacetate to liberate 6-carboxyfluorescein within the cytoplasm. However, this technique was not suitable for use in A431 cells due to a high rate of dye leakage from the cells, complicated further by apparent binding of the dye in the cell nucleus. Cytoplasmic trapping of pH-sensitive fluorophores linked to inert carrier molecules such as dextran permits rapid, continuous, and nondestructive pH, measurements over long time spans and apparently without ill effect on cell function. The method can be applied to a variety of mammalian cell types in culture, and so should be useful to assess the role of intracellular pH regulation in the initiation of normal (non-
transformed) cell growth. Unfortunately, the apparent pH, of fluorescein-dextran is such that little change in fluorescence is observed above pH 7.2–7.4. In preliminary experiments, we have found the intracellular pH of A431 cells to lie in this region, so we are unable to convincingly ascertain whether EGF alkalinizes the cytosol as our other data would suggest. This pH sensitivity limitation can be circumvented by using fluorescein derivatives or other fluorophores with higher pH, values, and such work is in progress. Using rough estimates of intracellular volume (3/106 cells) and intracellular buffering capacity (50 nmol·pH−1·liter−1, see Ref. 14) in A431 cells and, assuming all of the extra Na+ influx induced by EGF is exchanged for internal protons (50 nmol/106 cells at 10 min, Fig. 1), we would anticipate an intracellular pH rise on the order of a few tenths of a pH unit. Major metabolic alterations can be anticipated as a consequence of a pH change of this magnitude. If such a pH shift is demonstrable, it will be particularly interesting to investigate the biochemical mechanisms by which Na+/H+ exchange is activated.

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REFERENCES
30. Deleted in proof.