THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 20, Issue of October 25, pp. 12644-12653, 1983
Printed in U.S.A.

Activation of Na^+/H^+ Exchange by Epidermal Growth Factor Elevates Intracellular pH in A431 Cells*

(Received for publication, June 6, 1983)

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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. We have previously shown (Rothenberg, P., Glaser, L., Schlesinger, P., and Cassel, D. (1983) *J. Biol. Chem.* 257, 4883–4889) that EGF rapidly activates amiloride-sensitive net Na^+ influx in the A431 human epidermoid carcinoma cell line. We also described the presence of transmembrane, amiloride-sensitive Na^+/H^+ exchange in A431 cells using a new fluorescence technique for the measurement of intracellular pH (pH_i) based on the incorporation of fluorescein-dextran into the cell cytoplasm. The low pK_a of fluorescein (~6.4) prevented the direct assessment of the inferred, EGF-induced cytoplasmic alkalinization, mediated by stimulated Na^+/H^+ exchange.

In this paper, 4′,5′-dimethylfluorescein (pK_a 6.75) was coupled to dextran, allowing increased pH sensitivity of the fluorescence assay in the physiological range. Using this improved assay, basic features of pH_i regulation in A431 cells are documented, including the role of Na^+/H^+ exchange and Na^+-linked Cl^-/HCO_3^- exchange in acid extrusion. We directly demonstrate a rapid elevation of pH_i by addition of EGF as well as by serum in A431 cells. The pH_i increase is half-maximal at 5–10 ng/ml of EGF, is dependent on external Na^+, independent of external Ca^{2+}, and inhibited by millimolar amiloride. EGF and serum also enhance Na^+/H^+ exchange-mediated cytosolic acidification when the transmembrane Na^+ concentration gradient favors Na^+ efflux from the cells. An alkaline pH_i shift, caused by activation of Na^+/H^+ exchange, may be an important primary event in the mechanism of EGF action.

Increased Na^+ influx into mammalian cells is an early result of polypeptide growth factor activity (1–4). How this influx is stimulated, by what transmembrane pathway(s) it occurs, what physiological consequences result, and whether this ion flux is related to other cellular responses which follow mitogenic activation are largely unanswered questions. Recent observations are consistent with this Na^+ influx occurring through a transmembrane Na^+/H^+ exchange mechanism. Multiple effects on cellular biochemistry and physiology can be anticipated as a result of Na^+/H^+-mediated cytoplasmic alkalinization, suggesting that activated Na^+/H^+ exchange may play a central role in the mechanism by which growth factors cause quiescent cells to enter the cell cycle and proliferate (5).

Epidermal growth factor, perhaps the most intensively studied polypeptide growth factor, rapidly increases Na^+ entry in a variety of cell types *in vitro* (3, 4, 6). While this influx is electrically silent (4, 7), consistent with stoichiometric, one to one Na^+/H^+ exchange, other modes of electroneutral Na^+ influx, not involving H^+ movements, are possible (8). Inhibition of the Na^+ influx by the diuretic amiloride, a known antagonist of Na^+/H^+ exchange in other systems (9), strongly supports, but does not prove, activation of Na^+/H^+ exchange by EGF.[1] Furthermore, there is no direct evidence for a rise of intracellular pH resulting from EGF action.

In a recent study, we described the augmented Na^+ influx produced by EGF in the A431 epidermoid carcinoma cell line and documented the presence of amiloride-sensitive Na^+/H^+ exchange in these cells by a new fluorescence technique for determining pH_i (6). Although we were then unable to detect an EGF-provoked rise of pH_i in these cells due to a limitation of the fluorescence assay, those results did lend credence to the hypothesis that EGF-activated Na^+/H^+ exchange might increase pH_i and prompted us to extend the range of the fluorescence assay by using an alternate fluorophore. In this paper, we use this method to examine the role of Na^+/H^+ exchange in the pH_i regulation of A431 cells and demonstrate that EGF activation of Na^+/H^+ exchange leads to an increase of pH_i.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: dextran (average M_r = 5,000) (Pharmacia); 4,4-diisothiocyanostilbene-2,2′-disulfonic acid, ouabain, 5,5-dimethyl-2,4-oxazolidinedione, bovine insulin, gluconate salts, *N*-methyl-D-glucamine, Hepes (Sigma); bovine sera (KC Biologicals); 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (Fluka); polyethylene glycol 1500 (average M_r = 500–600), trimethyl ammonium (Fisher); sucrose ("ultrex") (Baker). Amiloride HCl was generously provided by A. W. Alberts of Merck, Sharp and Dohme Co. EGF was prepared by the method of Savage and Cohen (10). Purified platelet-derived growth factor was a generous gift of Drs. J. Huang and T. Deuel, Washington University School of Medicine. Fluorescein-dextran (average M_r = 40,000) was prepared essentially as described by DeBelder and Granath (11).

Cell Culture—The A431 cell line, obtained through Dr. C. F. Fox, University of California, Los Angeles, was maintained as previously described (6).

* This work was supported by National Institutes of Health Grant GM 18405, National Science Foundation Grant PCM 80 11 973, and a grant from the Monsanto Chemical Company. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Grant GM 02016. Portions of the material described herein were submitted as part of a thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] The abbreviations used are: EGF, epidermal growth factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH_i, intracellular pH; pH_o, extracellular pH; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 4-morpholineethanesulfonic acid.

Intracellular pH Measurements—The osmotic lysis technique (12) used to inject labeled dextran into the cytoplasm of A431 cells and the method of fluorimetric pH_i determination from confluent cell monolayers grown on small glass slides have been described previously (6). In most experiments, cells not grown to complete confluency yielded the most consistent results. Fig. 1, *A* and *B* demonstrate the diffuse cytoplasmic fluorescence which follows the osmotically induced release of labeled dextran from intracellular vesicles. Fig. 1, *C* and *D* show a similar field of cells allowed to take up fluorescein-dextran, without subsequent osmotic lysis. Note the punctate fluorescence indicative of the vesicular localization of the dye, compared to the uniform fluorescence in cells in which the dye has been released from endocytic vesicles by osmotic lysis. In the experiments reported here, the 4′,5′-dimethyl derivative of fluorescein replaced fluorescein as the pH-sensitive fluorophore. The synthesis of the dimethyl derivative and its covalent coupling to dextran is described below. Calibration of observed cellular fluorescence with pH_i is described under "Results." Fluorescence measurements were made at 37 °C with an Aminco SPF-500 spectrofluorimeter, using an excitation wavelength of 512 nm (1 nm band pass) and an emission wavelength of 540 nm (15 nm band pass). Light scattering contributed less than 1% of the total signal with confluent, well loaded cell monolayers. Control experiments with nonfluorescent, unloaded monolayers ruled out possible artifacts due to unanticipated light-scattering changes. Signal dampening was set for a 99% response within 10 s, a time constant much shorter than the time constant of the physiological events reported here. Solutions were exchanged by perfusing the volume of solution bathing the cells (~2.4 ml) with at least 4–5 volumes of fresh solution. Normal saline solutions were composed of: 110 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 25 mM glucose, 25 mM Hepes, at the pH indicated in each experiment. All solutions were passed through a 0.22-μm Millipore filter before use.

Synthesis of 4′,5′-Dimethylfluorescein Isothiocyanate—The preparation of 4′,5′-dimethylfluorescein as first described by Burton and Kurzer (13) was modified to yield 4′,5′-dimethylnitrofluorescein. Two-tenths mol of 4-nitrophthalic acid (Sigma), 0.4 mol of 2-methylresorcinol (Aldrich), and 0.2 mol of anhydrous, granular $ZnCl_2$ were melted together at 185 °C for 1 h and for 20 min longer at 200 °C. The cooled, glass-like product was powdered, boiled in 800 ml of 3% HCl for 15 min, filtered, washed with 500 ml of hot 3% HCl, washed with water, and dried to give crude 4′,5′-dimethylnitrofluorescein.

Reduction of the nitro group to an amine and recrystallization from HCl was carried out using the method of McKinney *et al.* (14). The partially purified 4′,5′-dimethylfluoresceinamine (2.5 gm) was dissolved in 30 ml of dry acetone and added during 30 min to a stirred 20-ml volume of dry acetone also containing 2.5 ml of thiophosgene (Baker). The suspension was stirred for 1 h at 25 °C and the product was filtered, washed with acetone, and washed with H_2O to remove HCl (15). The product was dried *in vacuo* for 18 h at 25 °C. Thin layer chromatography of this material on silica gel (Kieselguhr F254, Merck, Darmstadt) using chloroform:methanol:acetic acid (74:25:1, v/v/v) revealed the presence of the two expected isomers (16), together comprising about 85–90% of the spotted material.

A novel derivative of fluorescein, 2′,4′,5′,7′-tetramethyl fluorescein isothiocyanate was also prepared. The synthesis was similar to that just described for the dimethyl derivative, but 2,4-dimethylresorcinol was used instead of 2-methylresorcinol. 2,4-Dimethylresorcinol

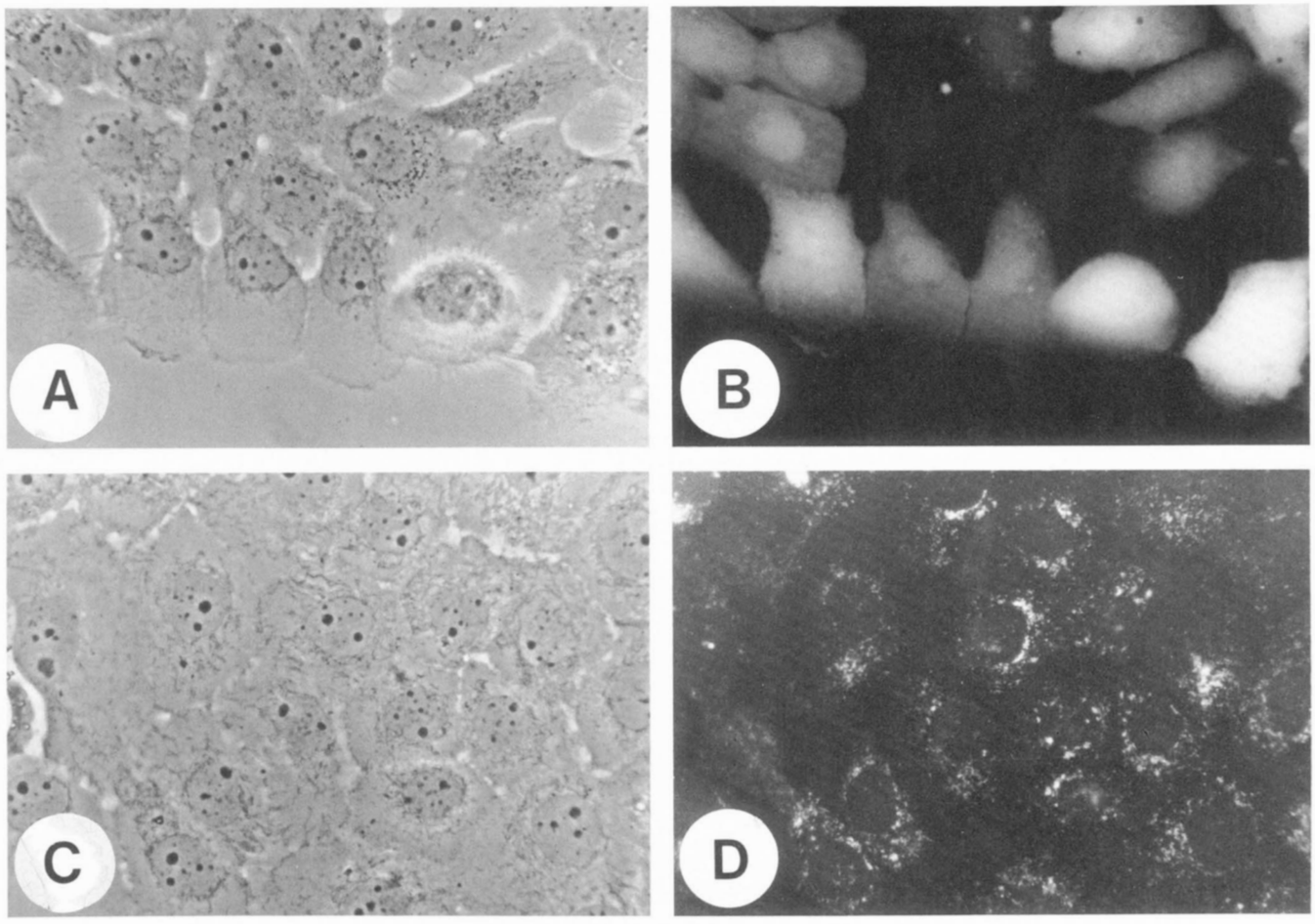

FIG. 1. **Release of dimethylfluorescein-dextran by osmotic lysis of pinosomes.** A431 cell monolayers were loaded with dimethylfluorescein-dextran and osmotically stressed as described under "Experimental Procedures" (*A* and *B*). *A*, phase contrast; *B*, epifluorescence image of the same field as in *A*. Bleaching during photographic exposure reduces the apparent fluorescence in *B*, but the intensity variations commonly observed among different cells is accurately depicted. A431 monolayers loaded with dimethylfluorescein-dextran (5 mg/ml) for 20 h and *not* osmotically stressed (*C* and *D*). *C*, phase contrast; *D*, epifluorescence image of the same field as in *C*.

was prepared as described by Baker *et al.* (17). The tetramethyl derivative was found to have a pKa of about 6.9 by fluorimetric titration, which is only marginally higher than the pK_a of dimethylfluorescein (pK_a = 6.75). Because there is no isosbestic point in the tetramethylfluorescein fluorescence spectra and due to the added difficulty of its preparation this derivative was not used in further work.

Preparation of Hydrazine-Dextran—Four g of dextran (M_r = 5000) was dissolved in 8 ml of water. Two mmol of sodium metaperiodate was added with continuous stirring, the pH maintained at 4.5 by adding 5 N NaOH. The solution of oxidized dextran was applied to a column containing 30 ml of Dowex anion exchange resin (AG2-X8) in the chloride form and the dextran eluted with distilled water. The eluate was added dropwise into 20 ml of 0.1 mol of hydrazine hydrochloride, pH 5.0. One h after the addition of dextran was complete, the solution pH was adjusted to 6.0 with NaOH. Four mmol of sodium cyanoborohydride in 4 ml of 1 M Mes buffer, pH 6.0, was then added and the solution was incubated for 16–20 h at room temperature under nitrogen. Subsequently, the pH was brought to 9.0 with NaOH, 2 mmol of sodium borohydride contained in 2 ml of 1 M potassium phosphate buffer, pH 9.0, was added, and the solution was further incubated for 5 h at room temperature.

The solution was added slowly into 3 volumes of 95% ethanol with shaking to precipitate the dextran. After settling, the supernatant was decanted and the sticky precipitate was dissolved in 20 ml of water and dialyzed (in Spectrapor No. 3 tubing, M_r = 3500 cutoff) at 4 °C against 4 liters of water for 36 h with two changes, and then lyophilized. The yield was 2.5–2.8 g. The colored reaction product of free amino groups with trinitrobenzenesulfonate (18) was used to estimate the presence of about 200–240 μmol of free NH_2 per g of dextran product. The hydrazine dextran was stored in a desiccator.

Coupling of Dimethylfluorescein Isothiocyanate to Hydrazine-Dextran—2.5 g of hydrazine-dextran was dissolved in 50 ml of dry dimethyl sulfoxide with gentle heating, 400 mg of dimethylfluorescein isothiocyanate was dissolved in 6 ml of dimethyl sulfoxide, and the two dimethyl sulfoxide solutions were combined and incubated for 15 min at room temperature. The dextran was precipitated by adding the dimethylsulfoxide solution to 200 ml of ethanol with rapid stirring. The precipitate was filtered with suction and washed with ethanol until the filtrate was clear. The product was dissolved in 20 ml of 1 M ammonium acetate, pH 8.0, and dialyzed against 2 liters of 0.1 M ammonium acetate, pH 8.0, at 4 °C overnight. Dialysis was continued against excess volumes of double-distilled water for 24 h with one change and the product then was lyophilized. Substitution of the free amino groups on the dextran with dimethylfluorescein isothiocyanate was nearly quantitative as assessed by fluorimetry. About 1 mol of dimethylfluorescein isothiocyanate was coupled per mol of dextran (average M_r = 5000). The predicted structure of dimethylfluorescein-dextran is shown in Fig. 2, together with the fluorescence emission spectra of this conjugate.

RESULTS

Cytoplasmic pH is regulated by transmembrane ion transport mechanisms which counteract metabolic acidification and passive H^+ influx across the plasma membrane. The review of Roos and Boron provides a comprehensive analysis of this subject (19). Using the fluorescence assay described under "Experimental Procedures," A431 cells were found to maintain intracellular pH by transport processes analogous to those in other cell types—mechanisms mostly understood through electrophysiological techniques employing microelectrodes suited to cells larger than cultured mammalian cells. Basic features of these regulatory processes are described in the following section. We then analyze in detail the influence of growth factors on pH_i and pH_i regulation.

Calibration of Intracellular pH—In previous studies with A431 cells (6), we used fluorescein-labeled dextran to assay pH_i. The fluorescence excitation spectrum of fluorescein has an isosbestic point, such that pH_i can be estimated unambiguously from the ratio of fluorescence intensity at two wavelengths (20). The higher pK_a of dimethylfluorescein-dextran (pK_a = 6.75) compared to fluorescein (pK_a = 6.4) is better suited for pH_i measurements under physiological conditions,

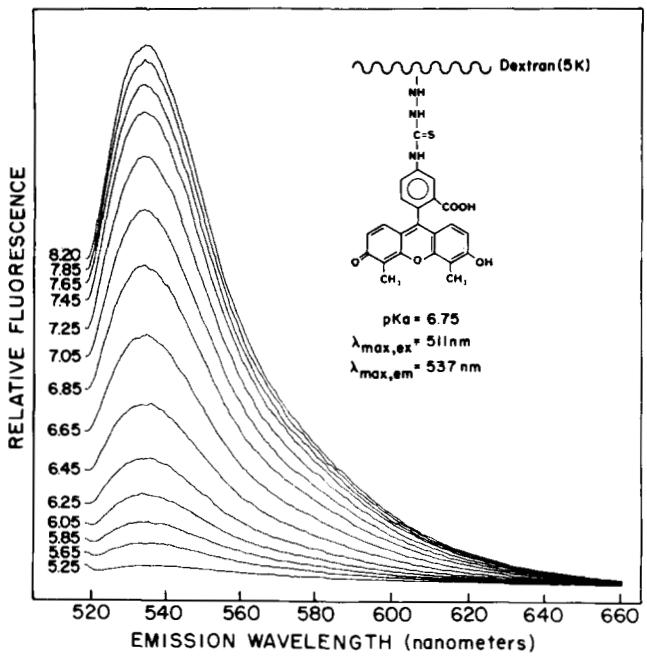

FIG. 2. **pH dependence of dimethylfluorescein-dextran fluorescence emission spectra.** Dimethylfluorescein-dextran (0.1 μg/ml) was dissolved in 150 mM KCl, 10 mM Hepes and the pH was adjusted with NaOH to the values indicated at the *left end* of each curve. The excitation wavelength was 511 nm.

but the fluorescence spectra of dimethylfluorescein-dextran lack an isosbestic point. Therefore, the fluorescence intensity must be independently calibrated with pH_i. Thomas *et al.* (21) used nigericin, a K^+/H^+ ionophore to equilibrate H^+ across the plasma membrane, when $[K_i^+] = [K_0^+]$. This technique has a potential disadvantage in that nigericin also equilibrates H^+ across the lysosomal membrane. Any dye present within lysosomes, at the normal intralysosomal pH of 5.0, will not contribute to the observed fluorescence under physiological conditions, but will significantly contribute to the observed fluorescence in the presence of nigericin (22). To calibrate the measurement of pH_i, we have made use of our previous observation that after 2 h of incubation in ouabain-containing saline, there is full activation of Na^+/H^+ exchange in A431 cells.

As shown in Fig. 3, in the presence of ouabain, the fluorescence intensity of dimethylfluorescein-dextran-loaded cells changes rapidly with pH_0, reaching stable values. The fluorescence intensity is plotted in Fig. 4 (*triangles*) and compared to the fluorescence titration of dimethylfluorescein-dextran obtained in solution (*solid line*); the data are in good agreement. Also shown in Fig. 3 is the effect of pH_0 on the fluorescence of cells not poisoned with ouabain. Such cells change pH_i much more slowly than ouabain-treated cells. Even after 30 min, when stable fluorescence intensities are reached (not shown), pH_i does not equilibrate with pH_0. This is expected from similar measurements of the relationship between pH_i and pH_0 in other cell types, as determined by various methods (23–25).

Control experiments show that dimethylfluorescein-dextran fluorescence is insensitive to the concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+} within physiologically relevant ranges.

Comparison of the ouabain method of pH_i calibration and the nigericin method in A431 cells suggests that in some cases up to 25% of the dye may be in a lysosomal compartment, in agreement with the data of Okada and Rechsteiner (12). The fraction of dye trapped in lysosomes following the dye-loading

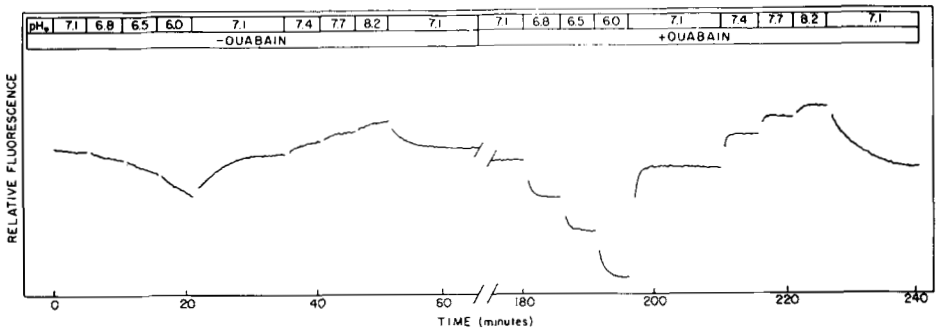

FIG. 3. **Effect of external pH on cellular fluorescence.** Measurements were made on confluent dimethylfluorescein-dextran-loaded A431 cell monolayers. First the cells were incubated in normal saline at the pH values indicated (0–65 min). At 65 min, ouabain was added in normal saline, pH 7.1. After 2 h of incubation, the cells were exposed to normal saline containing ouabain at the pH values indicated. Note that in the absence of ouabain pH_i changes modestly in response to external pH. But the cellular pH equilibrates with pH_i in ouabain-treated cells. A calibration of these observations on an absolute pH scale is shown in Fig. 4. The ouabain concentration was 0.5 mM and the external pH during each segment is indicated in the *box* at the *top* of the figure.

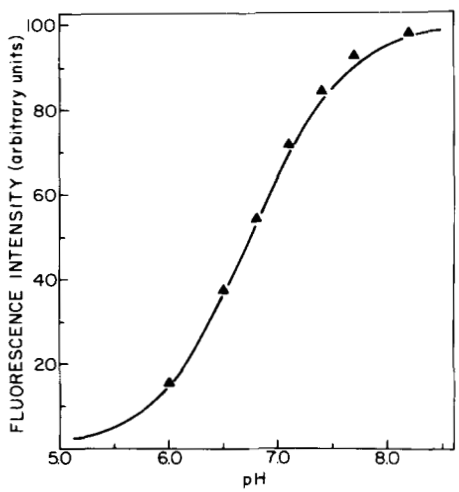

FIG. 4. **Calibration curve: cellular fluorescence *versus* pH.** Fluorescence intensities from ouabain-treated A431 cell monolayers (▲) were obtained from the stable levels observed at each level of external pH, as depicted in Fig. 2. The *solid line* is the titration curve of dimethylfluorescein-dextran in solution obtained from fluorescence data as in Fig. 1.

procedure may well vary in different cell types. The calibration using ouabain-treated cells is not affected by the lysosomal compartment, as determined in control experiments in which fluorescein-dextran was used to measure intralysosomal pH. No change of intralysosomal pH was found when cells were incubated for prolonged periods in ouabain-containing saline. Using this method, at pH_o 7.2 and 37 °C and in the absence of ouabain, the pH_i of A431 cells is 7.3 ± 0.1 (mean ± S.D., $n = 6$).

A primary pathway for H^+ flux across the plasma membrane of A431 cells after prolonged incubation in ouabain-containing saline is evidently through the Na^+/H^+ exchanger, because either the absence of Na^+ or the presence of 3 mM amiloride inhibits the rate of change of fluorescence with variations of pH_o by about 70% (results not shown). In essence, the intrinsic Na^+/H^+ exchanger can be exploited to equilibrate pH_i and pH_o in the same manner as the nigericin and high $[K_o^+]$ method, but the results are uncomplicated by lysosomal contributions.

Response of pH_i to Acid Loads—Acutely challenged with an acidification of the cytosol, the pH_i of A431 cells is rapidly restored to near normal levels. Fig. 5*a* demonstrates this recovery process following exposure to dissolved CO_2. At the *first arrow*, the Hepes-buffered saline, pH 7.2, was exchanged for a saline solution of the same pH, but without Hepes, buffered with HCO_3^- and equilibrated with 5% CO_2. The abrupt decline of pH_i was followed by a rapid, exponential recovery of pH_i back to its original level. Removal of the HCO_2^-/CO_2-buffered saline at the *second arrow* was followed by a large overshoot of pH_i, which rapidly returned back to the initial pH_i. This pattern of recovery and overshoot has been observed in squid giant axon (26), barnacle muscle fibers (27), and snail neuron (28) and the explanation for these events is similar. However, the rate of pH_i change during these processes is probably accelerated in A431 cells due to the greater surface to volume ratio of these small mammalian cells as compared to the aforementioned cell types which have much larger dimensions. Passive entry of membrane-permeant CO_2 is followed by hydration and dissociation to form H^+ and HCO_3^-, causing the initial drop of pH_i. The magnitude of the drop is a function of the intrinsic buffering capacity of the cytosol and intracellular organelles. The rise of pH_i in the continued presence of CO_2 is attributed to active acid extrusion (H^+ out or OH^- in) from the cytosol, as passive CO_2 and HCO_3^- fluxes may only cause further acidification. Removal of external CO_2 causes rapid exit of internal CO_2. The acid load is eliminated as H^+ and HCO_3^- reassociate and also leave as CO_2. Because proton equivalents had been actively extruded from the cytosol during the preceding recovery phase, the pH_i now rises above its initial value. The reader is referred to the work of Boron and De Weer (26) for a quantitative analysis of similar processes in the squid axon. The post-overshoot return of pH_i back down to its initial level has also been observed in other cells, but, in contrast to the proton extruding-acid recovery mechanisms, is less well understood, and we have not characterized it further.

The recovery process is dependent on external Na^+. When all external Na^+ is replaced by *N*-methyl-D-glucamine, recovery is absent (Fig. 5*b*). The external Na^+ concentration dependency of recovery from 5% CO_2 is plotted in Fig. 6. The initial rate of recovery is half-maximal at about 42 mM Na_o^+. Although the rate of recovery was only slightly inhibited by amiloride (~10% decrease at 3 mM amiloride, Fig. 5*c*), a known antagonist of Na^+/H^+ exchange, 80 μM 4,4-diisothiocyanostilbene-2,2′-disulfonic acid diminished the initial rate of recovery by about 70% (Fig. 5*d*). 4,4-Diisothiocyanostilbene-2,2′-disulfonic acid is the most potent of the stilbene derivatives known to block Cl^-/HCO_3^- exchange in erythrocytes (29). 4,4-Diisothiocyanostilbene-2,2′-disulfonic acid also inhibits Na^+-dependent Cl^-/HCO_3^- exchange in neurons

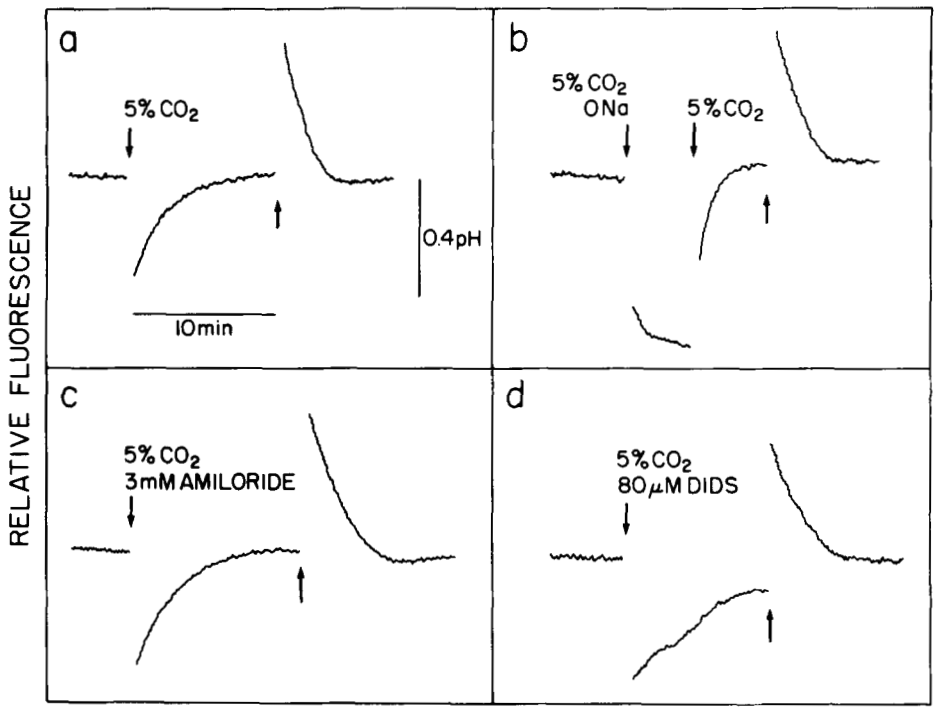

FIG. 5. **Recovery of pH_i following acidification by CO_2.** Fluorescence was measured in monolayers of A431 cells as described under "Experimental Procedures." At the *first arrow* in *a*, a normal saline solution buffered with 25 mM Hepes, pH 7.18, was replaced with a saline solution also pH 7.18 and of the same composition but lacking Hepes, buffered with HCO_3^-, and equilibrated with 5% CO_2. At the *second arrow* in *a*, the CO_2-saline was replaced with normal, Hepes-buffered saline. The experiment was repeated in *b*, but *N*-methyl-D-glucamine$^+$ replaced all Na^+ in the CO_2-saline (*first arrow*), then CO_2-free saline containing normal Na^+ concentration was restored (*second arrow*), and then the CO_2-saline was replaced with normal, Hepes-buffered saline (*third arrow*). Essentially the same results were obtained with trimethylammonium$^+$ as the Na^+ substitute. In *c* and *d*, 3 mM amiloride or 80 μM 4,4-diisothiocyanostilbene-2,2′-disulfonic acid, respectively, was added to the CO_2-saline. The *scale* indicating the calibration of fluorescence with pH_i shown in *a* is identical for *b*, *c*, and *d*.

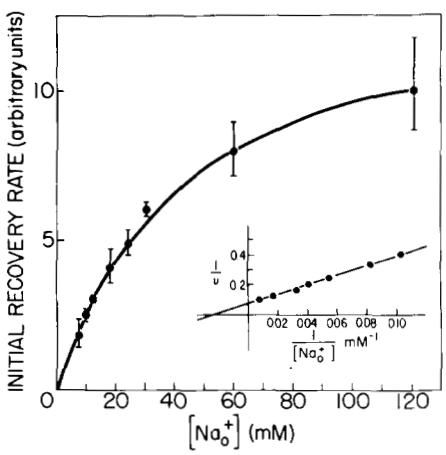

FIG. 6. **External Na^+ dependence of initial recovery rate from CO_2 acidification.** The experimental protocol is the same as that described for Fig. 4*a*. Na^+ was partly replaced with *N*-methyl-D-glucamine$^+$ to obtain the indicated concentrations. The pH_0 was 7.18. Initial rates were estimated from the nearly linear rates observed over the first 65 s of recovery. *Points* and *error bars* represent the mean ± S.E. of triplicate determinations. *Inset*, double reciprocal plot of the same data, with the line fit by linear regression, indicates an apparent half-maximal recovery rate at 42 mM.

(30). Similar inhibitory effects have been observed in A431 cells with 0.5 mM 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, another antagonist of Na^+-dependent Cl^-/HCO_3^- exchange (31).

If a major component of the acid extrusion mechanism in A431 cells responsible for recovery from CO_2 acidification is Na^+-linked Cl^-/HCO_3^- exchange, as these inhibitor experiments suggest, then the rate of recovery should have been diminished significantly in Cl^--depleted cells. Contrary to this expectation, the recovery rate observed in cells which had been preincubated in Cl^--free saline (assumed to deplete intracellular Cl^-, abolishing Cl^-/HCO_3^- exchange) was nearly the same (Fig. 7*a*) as recovery rates observed in normal Cl^--containing saline. Importantly, in Cl^--free saline, 3 mM amiloride inhibits recovery completely (Fig. 7*b*) whereas 80 μM 4,4-diisothiocyanostilbene-2,2′-disulfonic acid had only a slight inhibitory effect (Fig. 7*c*). The reversal of the relative inhibitory efficacy of amiloride and 4,4-diisothiocyanostilbene-2,2′-disulfonic acid in Cl^--free saline as compared to normal Cl^--containing saline indicates the presence of two Na_0^+-dependent, yet distinct mechanisms for acid recovery in A431 cells, an amiloride-sensitive process, independent of Cl^-, and most likely a Na^+/H^+ exchanger and a 4,4-diisothiocyanostilbene-2,2′-disulfonic acid-sensitive, Cl^--dependent process, probably similar to the Na^+-linked Cl^-/HCO_3^- exchange found in other cell types (33). Precedent for the operation of two such countertransport mechanisms operating in parallel is found in the studies of Aickin and Thomas on mouse soleus muscle (34).

Restoration of normal intracellular pH in A431 cells following the abrupt acidification caused by other permeant weak acids, such as acetate (Fig. 8) or 5,5-dimethyl-2,4-oxazolidinedione (not shown), follows a pattern like that observed in response to CO_2. Yet, recovery occurs in the nominal absence of HCO_3^- and the initial rate is at least 50% inhibited by 3 mM amiloride. Acid extrusion mediated by Na^+/H^+ exchange alone apparently suffices for complete pH_i homeostasis in this experiment. The effect of EGF also shown in this figure is described later. The relative contributions of Na^+/H^+- and Na^+-linked Cl^-/HCO_3^- exchanges in pH_i regulation in these cells during normal growth remains to be fully explored.

Influence of EGF and Serum on pH_i—Net Na^+ influx in A431 cells is stimulated by EGF or serum. Amiloride strongly inhibits this influx, while only slightly diminishing basal Na^+ entry (6). Because the plasma membrane of A431 cells bears an amiloride-sensitive Na^+/H^+ exchanger, these observations suggested the possibility of a hormonally induced pH_i elevation, mediated by activated Na^+/H^+ exchange.

The pH_i increases we observed upon exposure of A431 cells to EGF or serum at pH_0 6.8 are shown in Fig. 9, *a* and *b*. A latency of 1–3 min is routinely present prior to the sharp increase of cellular fluorescence. About 20 s are needed to exchange the volume of solution bathing the cells within the fluorimeter cuvette with 4–5 volumes of fresh solution, so that this lag is not an artifact. The basis for the lag period is

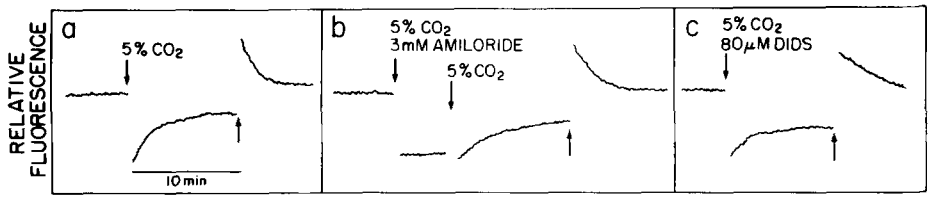

FIG. 7. **Recovery of pH_i from CO_2 acidification in the absence of Cl^-.** The experimental protocol is the same as that described in the legend of Fig. 5. All solutions were nominally Cl^--free with gluconate as the replacement anion. The concentration of calcium gluconate was 12 mM, calculated to yield a free Ca^{2+} concentration of 1.2 mM based on the Ca-gluconate binding constant (32). External Cl^- removal causes a slow pH_i rise which stabilizes after 30 min at pH about 0.3 unit greater than the initial pH_i. Monolayers were preincubated in Cl^--free saline for 1 h before initiating the measurements shown above. pH_o was 7.18 at all times. *DIDS*, 4,4-diisothiocyanostilbene-2,2′-disulfonic acid.

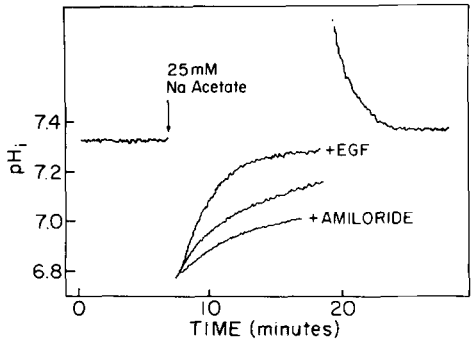

FIG. 8. **Recovery of pH_i from acetate acidification.** At the *first arrow*, normal saline of pH 7.18 was replaced with a saline solution, pH 7.18, of the same composition, but containing 25 mM Na acetate and lacking 25 mM NaCl (to maintain isotonicity). The subsequent recovery phase is a composite of three separate experiments. EGF (50 ng/ml) was added 5 min prior to the experiment and was also present during recovery (*top trace*). Amiloride (3 mM) was present only during recovery (*bottom trace*). No additions, *middle trace*. At about 20 min, the acetate-saline was replaced with normal saline.

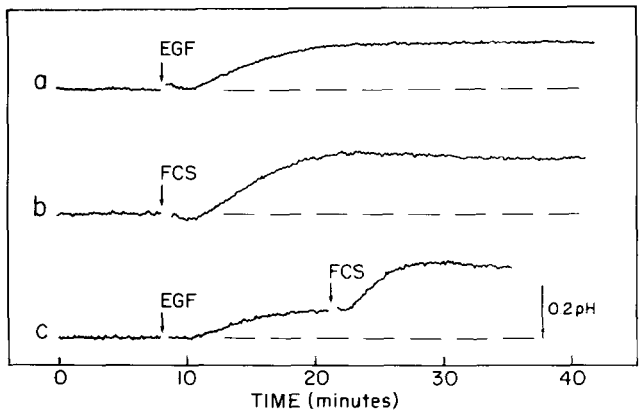

FIG. 9. **Effect of EGF and serum on pH_i.** Nearly confluent A431 monolayers were loaded with dimethylfluorescein-dextran as described under "Experimental Procedures," and incubated overnight in Dulbecco's modified Eagle's medium without HCO_3^- but with 25 mM Hepes, pH 7.3, also containing 2% (v/v) heat-inactivated fetal calf serum (*FCS*). Prior to the experiment, the cell monolayers were incubated for 4–6 h in serum-free Dulbecco's Modified Eagle's medium and then for about 1 h in normal saline solution, pH 6.8. EGF was added at 50 ng/ml (*a* and *c*). Fetal calf serum was dialyzed against saline solution (M_r = 1000 dialysis cutoff) before use and was added at 5% (v/v) (*b* and *c*). The records shown in *a*, *b*, and *c* are from different experiments.

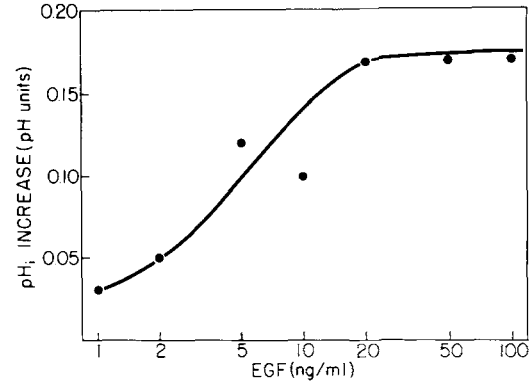

FIG. 10. **Dependence of pH_i increase on EGF concentration.** Monolayers of A431 cells were treated as described in Fig. 9, but were preincubated for 1 h prior to EGF addition in normal saline of pH 6.8 (rather than 7.18) to facilitate accurate measurement of small pH_i increases at the lowest EGF doses. *Points* represent single determinations. Similar results have been obtained in replicate experiments at pH_o = 7.18 (n = 2).

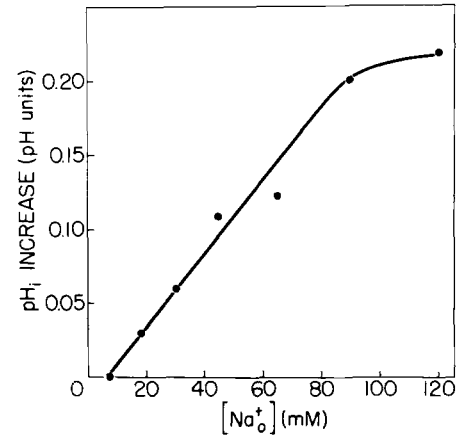

FIG. 11. **Dependence of the EGF-induced pH_i increase on Na_o^+ concentration.** A431 monolayers were treated as described in Fig. 9. *N*-methyl-D-glucamine$^+$ partly replaced Na^+ in the saline solutions to give the indicated concentrations. EGF was added at 50 ng/ml. Reducing Na_o^+ to levels below about 20 mM is associated with a slow pH_i decrease and data are corrected for this baseline drift.

uncertain. The maximal extent of fluorescence increase is dose-dependent (Fig. 10). The EGF concentration dependence for the pH_i increase is similar to that for EGF-induced $^{22}Na^+$ uptake (6), as well as for other reported effects of EGF on A431 cells, such as membrane ruffling (35), and tyrosine-specific protein kinase activity (36). Specificity of the EGF effect is also affirmed by the lack of effect of the mitogenic polypeptide platelet-derived growth factor on both $^{22}Na^+$ uptake and pH_i (results not shown). Similarly, bovine insulin (10 μg/ml) did not alter pH_i in these experiments.

The pattern of pH_i elevation caused by EGF or serum at an external pH of 7.2 is somewhat different from that shown

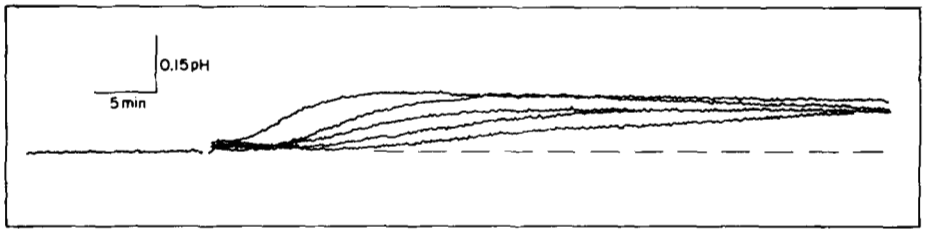

FIG. 12. **Amiloride inhibition of the EGF-induced pH_i increase.** A431 monolayers were treated as described in Fig. 8. This figure is a composite of five separate experiments at progressively increasing amiloride concentrations of 0, 0.1, 0.2, 1.0, and 3.0 mM (*top* to *bottom*). Amiloride was added from a 1 M stock in dimethyl sulfoxide prior to the addition (at the first break in the record) of 50 ng/ml of EGF. Dimethyl sulfoxide independently had no effect. Amiloride alone causes a slow, dose-dependent pH_i decrease (~0.025 pH unit/5 min at 3 mM) due to inhibition of basal Na^+/H^+ exchange and/or quenching of dimethylfluorescein-dextran fluorescence by drug taken up into the cells[2] and data are corrected for this baseline drift.

in Fig. 9. A few minutes after the initial increase, the pH_i slowly declines over the next 15–30 min to a level still above the basal level, but only about two-thirds as great as the maximal level. The alkalinization induced by EGF or serum persists for at least 1 h, at an extracellular pH of either 6.8 or 7.2.

Maximally effective levels of fetal calf serum elevate pH_i to a greater extent than the highest doses of EGF (Fig. 9). Dose-response experiments showed that maximal pH_i increases could be elicited at fetal calf serum levels of about 2% (v/v) (not shown). Similarly, serum increases $^{22}Na^+$ uptake to a greater degree than EGF (6). Also consonant with earlier $^{22}Na^+$ uptake studies, the combination of maximally effective doses of serum and EGF together produce a pH_i elevation greater than that induced by either mitogen added alone (Fig. 9*c*). The component(s) in serum responsible for influencing pH_i are as yet unidentified.

EGF increases the uptake of $^{45}Ca^{2+}$ in A431 cells (37). Although the mechanism and significance of this apparent influx is not known, previous studies had shown that EGF-stimulated $^{22}Na^+$ uptake was independent of external Ca^{2+} (6). Similarly, pH_i is elevated by EGF or serum in Ca^{2+}-free saline also containing 0.3 mM EGTA as in normal saline solution containing 1.8 mM Ca^{2+} (results not shown). This suggests that the pH_i rise is not secondary to hormone-triggered Ca^{2+} influx across the plasma membrane. Whether alterations of cytosolic Ca^{2+} activity (*e.g.* possibly resulting from hormonally induced mobilization of intracellular Ca^{2+} stores) influence the pH_i response remains to be determined.

The observed elevation of pH_i in response to EGF or serum is not artifactually compromised by intralysosomal pH changes registered by the fraction of dye retained within the lysosomes following the osmotic lysis procedure. Control experiments using cells which were permitted to pinocytose fluorescein-dextran, but which were not subsequently osmotically stressed, demonstrated no change in intralysosomal pH upon mitogen addition, as assessed by the 495:450 fluorescence ratio (22).

Fluorescence assays may be biased by unanticipated interferences. Conceivably, alterations in the concentrations of quenching metabolites secondary to hormone action could influence the fluorescence of cytoplasmic dimethylfluorescein-dextran. However, the following series of experiments renders such possibilities unlikely and provides further support for activated transmembrane Na^+/H^+ exchange as a locus of EGF action and as a mediator of the pH_i increase.

Effect of External Na^+ and Amiloride on EGF-induced Alkalinization—Net proton extrusion from the cytosol mediated through plasma membrane Na^+/H^+ countertransport requires a Na^+ concentration gradient directed inward. The magnitude of the transmembrane Na^+ gradient should influence the extent and perhaps the rate of the EGF-stimulated pH_i rise. Fig. 11 shows that the effect of EGF on pH_i is a function of the external Na^+ concentration. Although the levels of intracellular Na^+ are not known for all the extracellular Na^+ concentrations used, it is likely that the transmembrane Na^+ concentration gradient also declines with reductions of extracellular Na^+. The half-maximal response occurs at about 50 mM Na_o^+, a concentration similar to but higher than the Na_o^+ level yielding half-maximal rates of recovery from CO_2 acidification (Fig. 6). However, the latter recovery process probably involves a combination of Na^+/H^+ exchange and Na^+-dependent Cl^-/HCO_3^- exchange, so that the two half-maximal concentrations are probably not directly comparable. In addition, no pH_i elevation was elicited upon EGF or serum addition to cells which had been preincubated for 2 h in normal saline containing 0.5 mM ouabain (not shown).

The dependency of the EGF-induced alkalinization on external Na^+ is most likely related to Na^+/H^+ exchange and not to Na^+-linked Cl^-/HCO^- exchange because of the following. 1) The EGF-induced pH_i increase occurs in HCO_3^-/CO_2-buffered saline to an extent no greater than in nominally HCO_3^--free, Hepes-buffered saline (results not shown). 2) EGF almost doubles the initial rate of recovery from an acetate-induced acidification (Fig. 8) which occurs in a nominally HCO_3^--free saline solution, while having no significant effect on the rate of recovery from a CO_2-induced acidification (not shown). 3) Amiloride, a known antagonist of Na^+/H^+ exchange (9), blocks the EGF effect on pH_i (Fig. 12). The effect of amiloride is dose-dependent, with a half-maximal inhibition of the initial rate of EGF-induced pH_i rise at about 0.2–0.3 mM. Although the alkalinization rate induced by EGF decreases at higher amiloride concentrations, the maximal alkalinization is apparently about the same. However, since in the presence of amiloride and in the absence of EGF, there is some decrease in cellular fluorescence (representing slow acidification of pH_i and/or quenching by amiloride of dimethylfluorescein-dextran fluorescence), the absolute value of pH_i ultimately reached at different amiloride concentrations in Fig. 12 may not be the same. Note however that the effect of amiloride on the initial rate of EGF-induced alkalinization is unambiguous. Similar levels of amiloride were required to inhibit EGF or serum-stimulated $^{22}Na^+$ uptake (6). These latter results further support a close association of Na^+ influx and the pH_i rise.

Growth Factor Effect on "Reversed" Na^+/H^+ Exchange—Na^+/H^+ exchange may operate to acidify the cytosol if the Na^+ and H^+ concentration gradients favor Na^+ efflux from the cell. If intracellular Na^+ is first increased by inhibiting

[2] P. Rothenberg, L. Glaser, P. Schlesinger, and D. Cassel, unpublished observations.

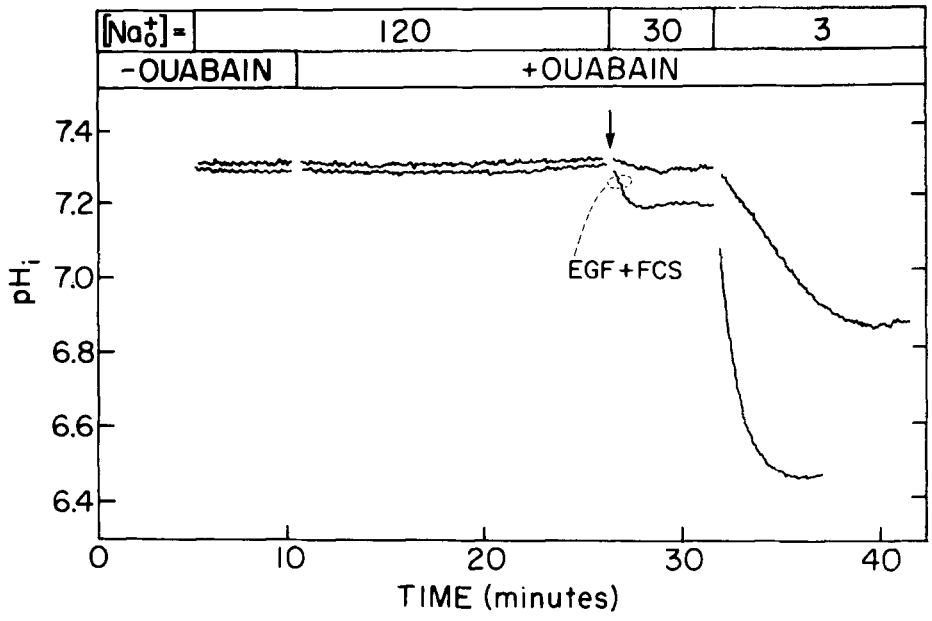

FIG. 13. **Effect of growth factors on pH$_i$ decrease mediated by Na$^+$/H$^+$ exchange.** A431 monolayers were treated as described in Fig. 9. Na$^+$ was partly replaced with *N*-methyl-D-glucamine$^+$ to obtain the indicated concentrations. The figure above is a composite of two separate experiments, superimposed for comparison. At about t = 10 min, the normal saline (120 Na$_o^+$) pH$_o$ 7.18, was exchanged for the same solution containing 0.5 mM ouabain. Ouabain was present in all solutions thereafter. At t = 26 min, the normal saline was replaced with a 30 mM Na$_o^+$ solution of the same pH, without growth factors (*upper trace*) or with the same solution containing both EGF (50 ng/ml) and fetal calf serum (*FCS*) (1%) (*lower trace*). At t = 31 min, the 30 mM Na$_o^+$ saline was replaced with a 3 mM Na$_o^+$ saline, also pH$_o$ 7.18.

the (Na$^+$, K$^+$)-ATPase with ouabain, acute reduction of extracellular Na$^+$ causes a rapid amiloride-sensitive decrease of pH$_i$ (6). EGF or serum increases the rate and extent of intracellular acidification during such a maneuver. In the experiments shown in Fig. 13, cells were incubated with ouabain in normal saline solution for 15.5 min and then exposed to 30 mM Na$_o^+$ with or without mitogens for the following 5 min. Little change in pH$_i$ (*top trace*, no added mitogens) or a small decrease in pH$_i$ (*lower trace*, EGF and serum added) was observed over this 5-min period. Preliminary experiments[3] had determined 15.5 min as time necessary for intracellular Na$^+$ to rise to a level such that no pH$_i$ shift occurred upon reducing extracellular Na$^+$ from 120–30 mM (at t = 26 min). Upon reducing the external Na$^+$ concentrations further to 3 mM (at t = 31 min), the expected acidification was observed. As evident in Fig. 13, exposure of the cells to serum and EGF markedly enhanced (about 6-fold) the rate of decline of pH$_i$. Similar results have been obtained with serum or EGF alone, with serum being more effective than EGF (3–4-fold and 2-fold effects, respectively). This result confirms our interpretation that EGF or serum activates Na$^+$/H$^+$ exchange and thus under normal conditions causes cytoplasmic alkalinization.

DISCUSSION

These experiments complement and parallel our earlier finding of EGF-stimulated ^{22}Na$^+$ uptake in A431 cells (6). The previous results demonstrated both amiloride-sensitive Na$^+$/H$^+$ exchange and mitogen-induced, amiloride-sensitive Na$^+$ influx, although definite evidence of growth factor-mediated cytoplasmic alkalinization could not be obtained due to the limitations of the fluorescent indicator. Extending the range of pH sensitivity of our intracellular pH assay by substituting dimethyl fluorescein for fluorescein now allows for the first time demonstration of the predicted pH$_i$ rise upon addition of EGF to these cells.

Our interpretation of the fluorescence increase observed upon mitogen addition as reflecting an increased pH$_i$ due to Na$^+$/H$^+$ exchange is supported by the similarities between ^{22}Na$^+$ uptake and the pH$_i$ response. Both ^{22}Na$^+$ uptake and pH$_i$ are increased to a greater extent by serum than by EGF and when simultaneously added these mitogens cause larger effects than either mitogen alone. Both ^{22}Na$^+$ uptake and the pH$_i$ elevation are independent of external Ca^{2+} and both responses are comparably inhibited by amiloride at millimolar concentrations. The dependency of the EGF-induced alkalinization on external Na$^+$ is expected for an uphill proton efflux driven by a downhill Na$^+$ influx following the transmembrane concentration gradient. EGF and serum enhance cytosolic acidification when the Na$^+$ gradient is reversed, consistent with an increased rate of Na$^+$/H$^+$ exchange.

Confidence in the validity of our fluorescence method of pH$_i$ determination stems from the similarity of the intracellular pH regulatory mechanisms observed here for A431 carcinoma cells and those mechanisms already documented through the use of electrophysiological techniques in other cell types, such as snail neuron and barnacle muscle. A431 cells recover a normal pH$_i$ after acute acid loading. Recovery is dependent on external Na$^+$, is inhibited by stilbene derivatives and amiloride, and probably involves the parallel operation of Cl$^-$/HCO$_3^-$ and Na$^+$/H$^+$ exchangers. Application of the fluorescence technique to other cultured cell types will help evaluate the generality of these features, as well as the role of intracellular pH regulation in other aspects of cell function.

Identification and characterization of the biochemical and physiological responses elicited after polypeptide growth factors bind to specific cell surface receptors is central to understanding the control of cell growth (38). EGF addition initiates a variety of effects in different cell types, such as increased nutrient transport (39), phosphatidylinositol turnover and Ca^{2+} uptake (37), protein kinase activity (40), cytoskeletal and morphological rearrangements (35, 41), and receptor clustering and internalization (42, 43), but knowledge is limited as to which of these are primary or secondary events or how such processes may act in concert to influence cell growth. Several defined growth factors and serum, in addition to EGF, rapidly stimulate monovalent cation fluxes. Induction of Na$^+$ influx through an amiloride-sensitive pathway occurs in hepatocytes (2, 44), fibroblasts (1, 3, 4, 45), epithelial cells (6,

[3] We have previously shown (6) that a 2 hr preincubation of A431 cells in normal saline with ouabain maximally activates Na$^+$/H$^+$ exchange, and we have also observed that the rate of acidification in such cells upon reducing extracellular Na$^+$ is uninfluenced by EGF. We therefore selected conditions that would elevate intracellular Na$^+$ without concomitant activation of Na$^+$/H$^+$ exchange.

7), and neuroblastoma (5), suggesting the generality of these phenomena. Several of these same cell types have demonstrable Na^+/H^+ exchange activity (46–48). Mitotic development triggered by fertilization of invertebrate oocytes is preceded by amiloride-sensitive Na^+/H^+ exchange and an ooplasmic pH_i rise of 0.3–0.5 pH units is observed by various techniques (for review, see Ref. 49). Whether a pH_i shift alone dictates the metabolic events necessary for cell growth remains to be determined. By analogy, if cytoplasmic alkalinization is found to be a ubiquitous element of the pre-replicative program of normal, nontransformed mammalian cells, similar questions will require resolution. For example, enzyme systems such as glycolysis, whose rates are enhanced at alkaline pH (50) and which are often activated in mitogenically stimulated or in transformed cells (51, 52), may be focal points with which to assess cause-effect relationships between pH_i and cell growth. We are encouraged to work in such directions by the recent report of Schuldiner and Rozengurt of a mitogen-induced pH_i rise in fibroblasts, as measured by 5,5-dimethyl-2,4-oxazolidinedione distributions (53), as well as our own recent fluorescence observations of a platelet-derived growth factor-induced rise of pH_i in the NR6 cell line, a mouse 3T3 variant lacking EGF receptors.[4] These observations will need to be extended to a variety of normal, quiescent cells to determine if a rise in pH_i upon mitogenic stimulation is an obligatory requirement for cell proliferation. A useful discussion of intracellular pH and growth control is found in the review of Gillies (54) to which the interested reader is referred.

The biochemical steps linking the initial EGF-receptor interaction and the activation of Na^+/H^+ exchange are unknown. EGF is not mitogenic for A431 cells and can inhibit growth after prolonged exposure (55, 56, but see Ref. 57). The high surface receptor density in these cells may however facilitate biochemical dissection of the linkage between the hormone receptor and Na^+/H^+ exchange, much as this feature has aided other studies of EGF action, such as tyrosine-specific protein kinase activity. Indeed, phosphorylation of membrane proteins modifies transmembrane ion transport in other cell systems (58), but it may be premature to speculate on a direct connection between kinase activity and Na^+/H^+ exchange. Hormones other than EGF may also influence the regulation of pH_i through Na^+/H^+ exchange, e.g. insulin may stimulate Na^+/H^+ exchange in frog skeletal muscle[5] (59). Through intracellular pH, Na^+/H^+ exchange may influence the expression of differentiated cell function. Perhaps other reported effects of EGF apart from the promotion of cell growth, such as modulating the synthesis of growth hormone and prolactin in pituitary cells (60, 61) and induction of specific enzymes in other cell types (62), are also related to pH_i changes.

Acknowledgments—We are indebted to Dr. Xingin Zhuang for expert assistance in the dye synthesis, to Carol Boyd for maintaining the A431 cells, and to Sophie Silverman and Suzanne Winkler for typing the manuscript.

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Activation of Na+/H+ exchange by epidermal growth factor elevates intracellular pH in A431 cells.

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J. Biol. Chem. 1983, 258:12644-12653.