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Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: Coupling to ribosomal protein S6 phosphorylation

(growth control/mitogen action/ionic flux/pH regulation/thrombin)

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ABSTRACT **Chinese hamster lung fibroblast cells (CCl39) enter the G_0/G_1 nonproliferative state after serum deprivation. In this report, we show that reinitiation of DNA synthesis by serum or the combination of purified human thrombin and insulin (1–10 μg/ml) is preceded by very early stimulation of ionic fluxes (Na^+/Rb^+) and protein phosphorylation (27,000 daltons, 62,000 daltons, and the ribosomal S6 proteins). The potentiating action of insulin on thrombin-stimulated DNA synthesis is also observed on thrombin-stimulated Na^+ influx, Rb^+ influx, and protein S6 phosphorylation. Moreover, we demonstrate that CCl39 cells possess a Na^+/H^+ exchange system sensitive to amiloride. Half-maximal inhibition of growth factor-activated Na^+ influx and Na^+-dependent H^+ efflux is obtained with 3–10 μM amiloride. Two lines of evidence indicate that the extrusion of H^+ via the activation of the Na^+/H^+ exchanger is coupled to protein S6 phosphorylation: serum-stimulated phosphorylation is blocked by (*i*) amiloride at a concentration that abolishes serum-stimulated Na^+ influx and (*ii*) protonophores that acidify the cell interior. The present data support the idea that the regulation of intracellular pH is a key event in the mechanism of growth factor action.**

Control of eukaryotic cell proliferation is among the major problems in biology that remain poorly understood. Polypeptide growth factors and hormones have been shown to play a crucial role in the regulation of cell division (1, 2). However, the molecular mechanisms by which these regulatory peptides act to stimulate cell metabolism, protein synthesis, DNA replication, and subsequent cell division are largely unknown.

Marked alterations in ionic fluxes are one of the earliest biochemical events detected after addition of serum or purified growth factors to arrested cells (3). Stimulation of Na^+ influx seems ubiquitous; it has been observed in various cell types activated for cell division (3–7). Closely associated with the increase in Na^+ uptake is activation of the Na^+/K^+ pump leading to increased K^+ influx (8).

Protein phosphorylation is another rapid biochemical change associated with growth factor action (9–11).

In light of these different observations, an obvious question is that of the relationship between these early events and mitogen action. Indeed, it is not known whether the monovalent ionic fluxes represent one of the initial triggers of mitogen action or secondary events to early metabolic activation. It is also not clear whether protein phosphorylation is coupled to growth factor-activated Na^+ influx.

To approach these questions, we have used a Chinese hamster lung fibroblast line, CCl39, capable of growing in a chemically defined medium (12) and reversibly entering the nonproliferative G_0/G_1 state (13). From this line, we have derived stable variants altered either in metabolic pathways (13, 14) or in growth factor dependence for DNA synthesis (15). We now report that CCl39 cells possess an amiloride-sensitive Na^+/H^+ exchange system that is activated by growth factors. We found that Na^+ influx and H^+ efflux are sensitive to the same range of amiloride concentrations. Moreover, we showed that amiloride blocks both growth factor-stimulated Na^+ influx and growth factor-stimulated ribosomal protein S6 phosphorylation.

This demonstration of the existence of a link between activation of the Na^+/H^+ exchanger and protein phosphorylation and the observation that acidification of the cell with weak acids also abolishes growth factor-activated S6 phosphorylation support the idea that regulation of intracellular pH is a key event in the mechanism of growth factor action.

MATERIALS AND METHODS

Materials used for these studies were obtained from the following sources: human thrombin (3,000 NIH units/mg) and bovine pancreas crystalline insulin (23.6 international units/mg) were from Sigma; amiloride [3,5-diamino-*N*-(aminoiminomethyl)-6 chlorpyrazinecarboxamide] was a gift from Merck Sharp & Dohme.

Cells and Culture Conditions. The Chinese hamster lung fibroblast line CCl39 (ATCC) was maintained in Dulbecco's modified Eagle's (DME) medium (GIBCO)/5% fetal calf serum supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml). Unless otherwise specified, cells were arrested in G_0/G_1 essentially as described (13): confluent cell monolayers were washed twice with serum-free medium and incubated for 30 hr in serum-free DME medium/Ham's F12 medium (1:1). Cells were maintained at 37°C in 95% air/5% CO_2.

[^{3}H]Thymidine Incorporation. For measurement of DNA synthesis, G_0/G_1-arrested cell cultures (serum-starved) were incubated for 24 hr with 3 μM [*methyl*-^{3}H]thymidine (1 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) in the presence of various concentrations of serum. The cells were then washed with phosphate-buffered saline at 0°C and fixed with 10% trichloroacetic acid (30 min, 0°C). Acid-precipitable material was solubilized in 0.1 M NaOH and radioactivity was assayed by liquid scintillation spectrometry.

Measurement of Na^+ and Rb^+ Uptake. Confluent cells cultivated in 35-mm dishes and arrested by serum starvation (30 hr) were washed twice with phosphate-buffered saline. Cells were incubated at 37°C with 0.9 ml of DME medium/20 mM Hepes. Serum, purified growth factors, and amiloride were added 1 min before initiating uptake. Uptake was initiated by addition of 0.1 ml of $^{22}Na^+$ (20 μCi/ml) or $^{86}Rb^+$ (5 μCi/ml). Uptake was stopped by rapidly washing cell monolayers at room

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Abbreviation: DME medium, Dulbecco's modified Eagle's medium.

temperature four times with 1-ml portions of saline/145 mM choline chloride/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/25 mM Hepes/Tris base, pH 7.4. Cell monolayers were solubilized in 0.1 M NaOH, and radioactivity was assayed by liquid scintillation counting. For Na^+ uptake, ouabain was present at 5 mM to prevent Na^+ exit via the Na^+/K^+ pump. In some experiments, the NaCl concentration in the DME medium was reduced to 14 mM and the osmolarity was maintained constant with choline chloride.

Measurements of Proton Release. Proton efflux was measured on cell suspensions essentially as described by Moolenaar *et al.* (16). Confluent CCl39 cells were detached from 100-mm dishes with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline by gentle pipetting. Cells were incubated in Na-free medium (130 mM choline chloride/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/20 mM Tris·HCl, pH 7.40, at 37°C for 30 min. Approximately 10^7 cells were centrifuged and suspended in 10 ml of 130 mM choline chloride/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/0.2 mM Tris·HCl, pH 7.40. The cell suspension was stirred at 30°C and acid production was monitored with a pH electrode coupled to a radiometer titration stand. pH was automatically maintained constant at 7.40 by titrating with 5 mM KOH/140 mM choline chloride.

Protein Phosphorylation. G_0/G_1-arrested cells in 35-mm dishes were washed twice with incubation medium (phosphate-free DME medium/20 mM Hepes, pH 7.4). Prior to growth factor stimulation, the cells were incubated for 45 min at 37°C with 1 μM $^{32}P_i$ (100–200 μCi/ml) to label intracellular ATP. Cells were then incubated for 15 min with serum or growth factor. Phosphorylation was terminated by washing the cell monolayer three times with phosphate-buffered saline and solubilizing the cells with $NaDodSO_4$ gel electrophoresis sampling buffer (2% $NaDodSO_4$/10 mM Na phosphate, pH 6.8). When growth factor-stimulated protein phosphorylation was carried out at various external Na^+ concentrations, the 45-min ^{32}P-cell labeling was performed in standard phosphate-free DME medium.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Total cell proteins labeled with ^{32}P were reduced with 0.1 M dithiothreitol and analyzed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis under the conditions described by Laemmli (17). The same amount of protein was added to each lane.

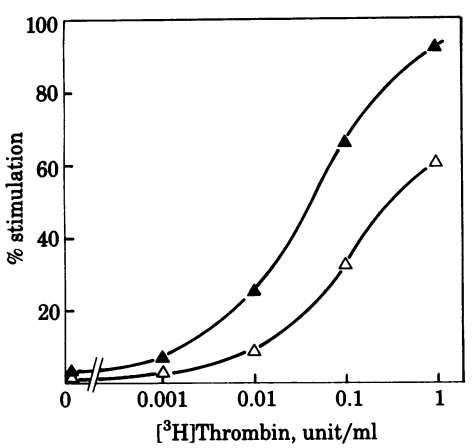

FIG. 1. Potentiating effect of insulin on human thrombin stimulation of DNA synthesis. Stimulation of DNA synthesis reinitiation in growth-arrested cells was evaluated as percentage of [^{3}H]thymidine incorporation relative to that in the presence of 10% fetal calf serum. △, α-thrombin alone; ▲, α-thrombin/insulin (10 μg/ml). The percentage of labeled nuclei obtained with 10% fetal calf serum is 60 ± 5%.

RESULTS

Correlation Between Late and Early Events Stimulated by Growth Factors. CCl39 cells arrested by serum starvation invariably reinitiate DNA synthesis 10–12 hr after addition of either serum or purified growth factor (12, 13). DNA replication (late events) is preceded by immediate stimulation (no detectable lag) of the Na^+/K^+ pump, as measured by $^{86}Rb^+$ influx. The stimulation of these two temporally separate events shows a remarkable correlation with increases in the concentration of serum (data not shown). We have previously shown that purified growth factors can replace serum, not only for DNA synthesis reinitiation but also for continuous proliferation of CCl39 cells (12). Insulin alone is not mitogenic but it strongly potentiates the actions of fibroblast growth factor, platelet-derived growth factor, and thrombin. The effect of insulin on the action of purified human thrombin on DNA replication of arrested CCl39 cells is shown in Fig. 1. Insulin substantially increases thrombin efficiency, as indicated by the leftward shift of the dose–response curve, and it also appears to increase the maximal effect of thrombin on DNA synthesis.

Comparison of the dose–response curve for stimulation of DNA synthesis (Fig. 1) with that for Na^+ influx (Fig. 2*A*) by purified thrombin indicates that half-maximal and maximal effects are obtained with similar concentrations of the growth fac-

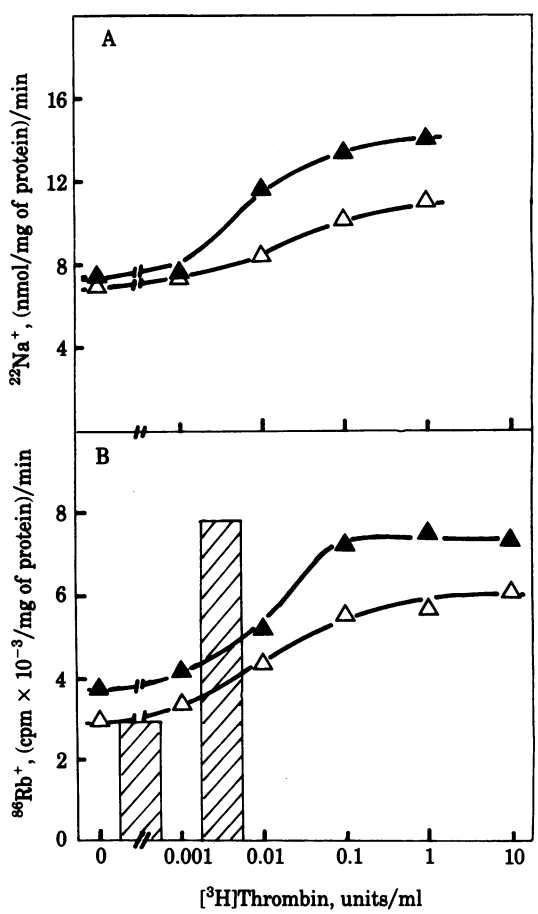

FIG. 2. Growth factor-stimulated ionic fluxes in G_0-arrested CCl39 cells. $^{22}Na^+$ (*A*) and $^{86}Rb^+$ (*B*) uptake rates were measured after stimulation by various concentrations of α-thrombin (△) or α-thrombin/insulin (10 μg/ml) (▲) over a 5-min incubation period. The left-hand hatched bar represents the basal initial rate of Rb^+ influx (no growth factor) and the right-hand one represents the serum-stimulated (10% fetal calf serum) rate of Rb^+ influx. Note the similar stimulation of Rb^+ influx by the combination α-thrombin/insulin and by fetal calf serum.

tor. Furthermore, the potentiating effect of insulin is similarly observed for early stimulation of the Na^+ influx rate by thrombin (Fig. 2*A*). Stimulation of Rb^+ influx parallels that of Na^+ (Fig. 2*B*). This result is consistent with the well-documented regulatory role of internal Na^+ concentration on Na^+/K^+ ATPase (8).

Serum or the combination of purified growth factors, human α-thrombin/insulin, stimulates phosphorylation of a common set of proteins (see below), including ribosomal protein S6 (unpublished). Likewise, for this early biochemical change (detected 5–15 min after exposure to growth factor), we have noticed a potentiating action of insulin on the level of phosphorylation of S6 and a 27,000-dalton protein (data not shown).

The Growth Factor-Stimulated Na^+ Influx Is Amiloride Sensitive. Previously, we have identified silent fast Na^+ channels in the plasma membrane of CCl39 cells (18). These channels can be activated by a combination of neurotoxins (sea anemone toxin II/veratridine). When activated, these Na^+ channels are specifically inhibited, as those of excitable cells, by tetrodotoxin (ref. 18; half-inhibition $ID_{50} = 30 \pm 10$ nM). We have shown that serum stimulation of Na^+ influx in quiescent CCl39 cells is not affected by tetrodotoxin (18) but is blocked by amiloride (Fig. 3). This result suggests that growth factors activate a precise route for Na^+ entry unrelated to the tetrodotoxin-sensitive Na^+ channels. At low external Na^+ concentration (14 mM), half-maximal inhibition of serum-stimulated Na^+ and Rb^+ influxes is obtained with 10 μM amiloride. High concentrations of the inhibitor (0.1–0.5 mM) are required to totally abolish growth factor-activated Na^+ influx.

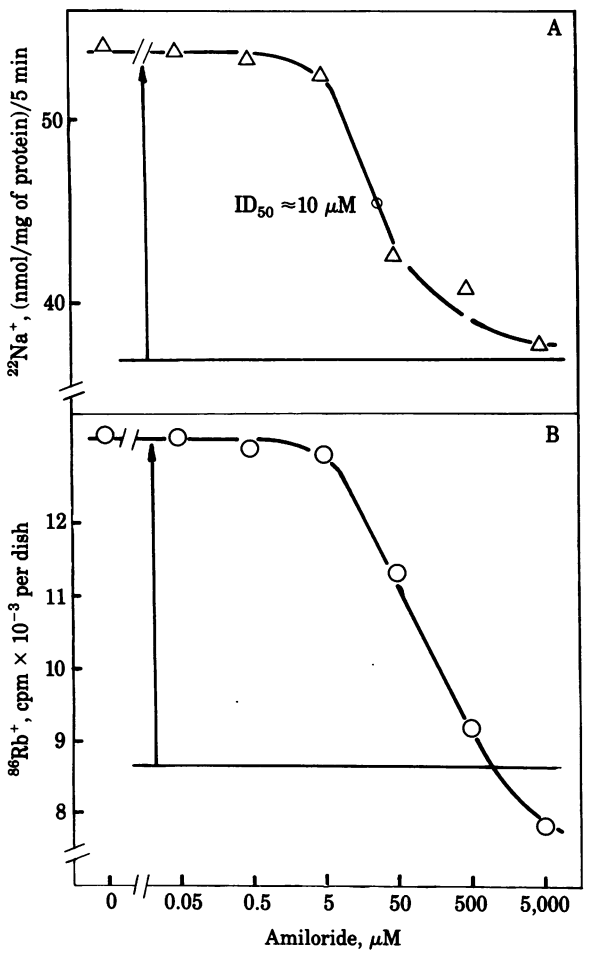

FIG. 3. Effect of amiloride on serum-stimulated Na^+ (*A*) and Rb^+ (*B*) influx rates in CCl39 arrested cells. Cells were arrested and influx rates were measured as described in *Materials and Methods* and Fig. 2. The Na^+ concentration of the uptake medium was 14 mM and the osmolarity was maintained with choline chloride. Cells were stimulated by 10% fetal calf serum (↑; dialyzed against 0.14 M choline chloride). In three independent experiments with duplicate dishes, the amiloride concentration inhibiting 50% of serum-stimulated Na^+ influx was found to be 10 ± 5 μM. Amiloride solutions were prepared from a stock solution 1 M in dimethyl sulfoxide. Horizontal line indicates basal uptake value.

Identification of a Na^+/H^+ Exchange System in the Plasma Membrane of CCl39 Cells. Amiloride has been shown to inhibit Na^+ uptake and Na^+/H^+ exchange in various differentiated cell types (19–22). This property, together with the fact that amiloride blocks growth factor-activated Na^+ influx in CCl39 cells, prompted us to analyze the movement of protons in these cells. We found that CCl39 cells incubated in Na^+-free medium release H^+ into the external medium at a constant rate (Fig. 4 *Inset*). Addition of 20 mM NaCl, creating an inwardly directed Na^+ gradient, immediately stimulated the rate of H^+ efflux ≈4-fold. This Na^+-dependent proton release (only Li^+ can replace Na^+) is saturable with an apparent K_m for Na^+ of ≈12 mM (unpublished). This Na^+ effect is completely inhibited by amiloride without any significant alteration of the basal level. Half-maximal inhibition of the Na^+-dependent H^+ efflux was obtained with 3 μM amiloride (20 mM NaCl, pH 7.40; Fig. 4). This dose response is similar to that observed with serum-stimulated Na^+ influx (Fig. 3*A*), suggesting the existence of a tight coupling between growth factor-stimulated Na^+ influx and proton release in CCl39 cells.

Growth Factor-Stimulated S6 Phosphorylation Is Blocked by Amiloride. Serum, or any combination of purified growth

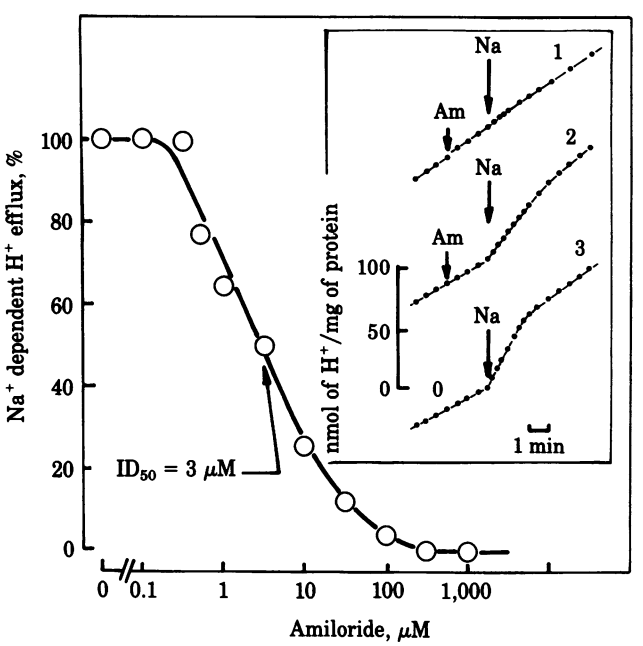

FIG. 4. Effect of amiloride on Na^+-dependent H^+ extrusion in CCl39 cells. Confluent cells were suspended and depleted of Na before treatment with amiloride. (*Inset*) Acid production as a function of time, as measured by titration with 5 mM KOH. Amiloride (Am) at various concentrations was added 2 min before addition of 20 mM NaCl, all solutions being previously adjusted to pH 7.40. Curves: 1, 0.1 mM amiloride; 2, 3 μM amiloride; 3, no amiloride. Initial rates of Na^+-dependent H^+ release were determined as differences in acid production rates before and after NaCl addition. The dose–response curve represents the effect of amiloride on initial rates of Na^+-dependent H^+ release relative to control (no amiloride). Data from three independent experiments were pooled; 100% ranged from 22 to 34 nmol of H^+ released per mg of protein per min.

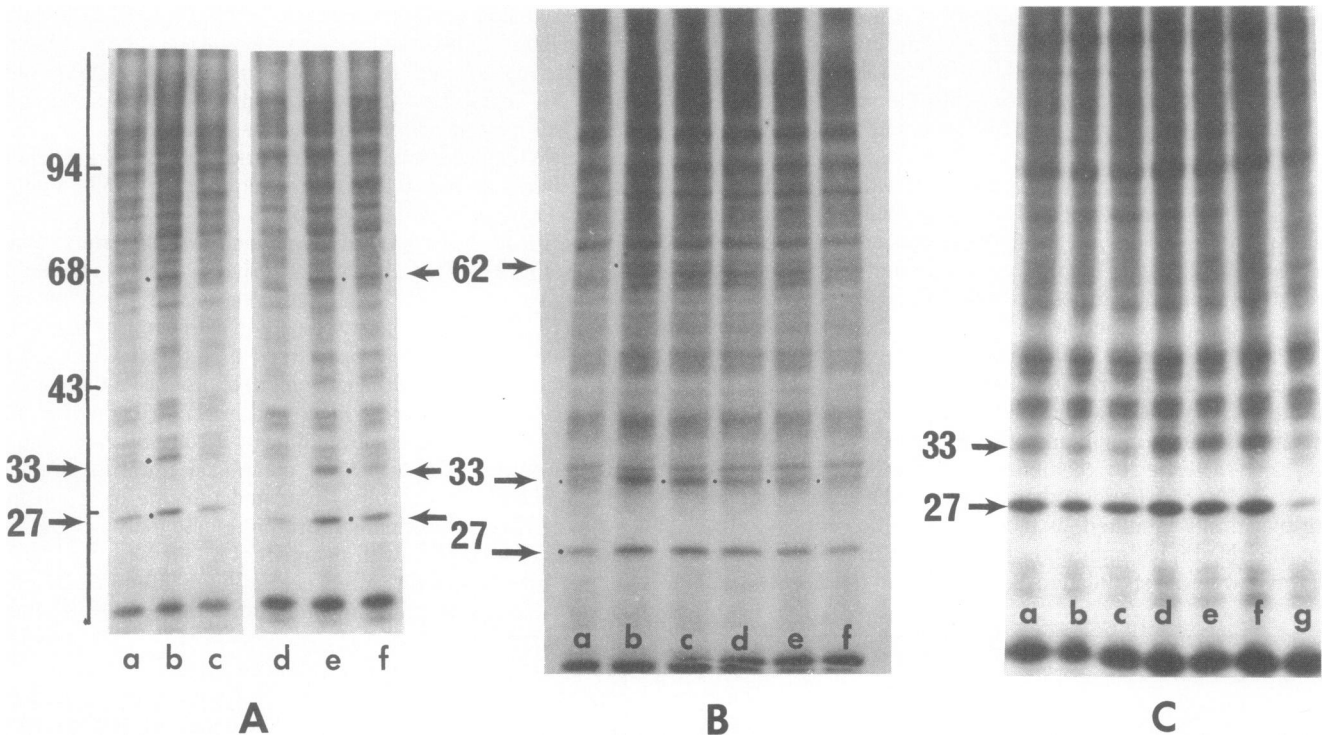

FIG. 5. Effect of amiloride and metabolic inhibitors on serum-stimulated protein phosphorylation in quiescent CCl39 cells. ^{32}P-Labeled serum-stimulated cells were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. (*A*) Serum-stimulated phosphorylation at two external Na^+ concentrations: 1.4 mM (lanes a–c) and 70 mM (lanes d–f). Lanes: a and d, unstimulated (0.05% dimethyl sulfoxide); b and e, stimulated by 10% dialyzed fetal calf serum in the presence of 0.05% dimethyl sulfoxide; c and f, stimulated by 10% serum in the presence of 0.5 mM amiloride. The amiloride-sensitive 33,000-dalton phosphoprotein has been identified as ribosomal protein S6 (unpublished). (*B*) Dose response of 10% fetal calf serum-stimulated phosphoprotein to various concentrations of amiloride at an external Na^+ concentration of 14 mM. Lanes: a, unstimulated; b–f, stimulated by 10% serum in the presence of various concentrations of amiloride: c, 0.15 mM; d, 0.5 mM; e, 1.5 mM; f, 5 mM. (*C*) Effect of various inhibitors on serum-stimulated phosphorylation of the 33,000-dalton protein. Cells were incubated for 5 min with the inhibitors and then stimulated with 10% serum for 15 min. Lanes: a and b, cells were treated with 0.025 and 0.25 mM, respectively, carbonyl cyanide *m*-phenylhydrazone; c, cells were treated with 2 mM dinitrophenol; d and e, cells were treated with oligomycin at 1 and 10 μg/ml, respectively; f, cells received no inhibitor; g, cells were not stimulated.

factors capable of reinitiating DNA synthesis of arrested CCl39 cells, stimulates phosphorylation of a common set of polypeptides. The major changes involve phosphopeptides of apparent molecular sizes 27,000, 33,000, and 62,000 daltons (Fig. 5*A*). The 33,000-dalton phosphoprotein was identified as ribosomal protein S6, based on its acid solubility (23) and its comigration in two-dimensional polyacrylamide gel (pH 5.5/NaDodSO$_4$) with rat liver ribosomal protein S6 (unpublished).

If stimulation of Na^+ influx or H^+ efflux is indeed coupled to growth factor-activated phosphorylation, amiloride, the specific blocker of the Na^+/H^+ exchange system, should inhibit phosphorylation. Fig. 5 *A* and *B* shows that, indeed, phosphorylation of ribosomal protein S6 (the 33,000-dalton peptide) is inhibited by 0.5 mM amiloride, a concentration that abolishes growth factor-stimulated Na^+ influx. Because of the competition between amiloride and Na^+, the amiloride inhibition of S6 phosphorylation, as well as that of minor phosphopeptides, seen at an external Na^+ concentration of 1.4 mM is less pronounced at higher Na^+ concentrations (70–140 mM; Fig. 5*A*). This finding suggests that S6 phosphorylation is coupled to cationic movement across the plasma membrane but does not indicate whether increases in Na^+ influx, H^+ efflux, or both are required to trigger phosphorylation. To further characterize this point, we analyzed the effects of weak acids known to dissipate proton gradients (dinitrophenol and carbonyl cyanide *m*-chlorophenyl hydrazone). Fig. 5*C* shows that the two protonophores abolish serum-stimulated phosphorylation of S6. In contrast, oligomycin, which, like the two protonophores, inhibits respiration-derived ATP, but does not collapse H^+ ion gradients, does not prevent S6 phosphorylation. These data point out that a decrease in intracellular pH is a severe limiting step to growth factor-stimulated ribosomal protein S6 phosphorylation.

DISCUSSION

CCl39 cells possess a growth factor-activatable Na^+ porter. This system, which is specifically blocked by amiloride, is pharmacologically distinct from the tetrodotoxin-sensitive Na^+ channels present in these cells (18). We now report that CCl39 cells possess, in addition, a Na^+-dependent acid extrusion system. Two lines of evidence indicate that the acid release corresponds to H^+ ion efflux: (*i*) the Na^+-dependent acid extrusion is unchanged in a medium depleted of glucose and oxygen and (*ii*) the cells, reversibly, take up H^+ from the external medium when the Na^+ gradient is reversed (140 mM Na^+_{in}–0 mM Na^+_{out}; unpublished). That serum or growth factor-activated Na^+ influx is electroneutrally coupled to simultaneous efflux of protons out of the cell is supported by the following observations: (*i*) proton efflux is strictly dependent on Na^+ (or Li^+), (*ii*) the direction of the Na^+ gradient directs the proton flux outward or inward, and (*iii*) amiloride blocks growth factor-activated Na^+ influx and proton release with a similar dose–response curve (ID_{50} = 3–10 μM). The latter property agrees with the known ability of amiloride to block electroneutral Na^+/H^+ exchange in various differentiated cell types: mouse soleus muscle (19), dog kidney cells (20), frog muscle cells (21), and neuroblastoma cells (16).

A striking observation is the ubiquity of rapidly increased Na^+ influx in response to activation of growth or differentiation (or both) of various cell systems. It has been observed in sea urchin eggs (7), hepatocytes (6), neuroblastoma cells (22), lymphocytes (23, 4), frog muscle cells (21), platelets (24), and fibroblasts (3, 8). Therefore, it is not surprising that a variety of growth factors (e.g., thrombin, fibroblast growth factor, platelet derived growth factor) that bind to their own receptor sites in a given cell line elicit a common early ionic response. Two models could account for this convergent phenomenon. In model 1, activation of Na^+ influx and H^+ release are secondary to activation of the intermediate metabolism. In model 2, the Na^+/H^+ exchange system serves as an initial transmembrane signaling device upon activation by external agents. In model 1, the interaction of growth factors with their receptors delivers (a) signal(s) that activates enzyme phosphorylation–dephosphorylation and therefore metabolism. Increase in intracellular H^+ production as a consequence of metabolic activation will activate the Na^+/H^+ exchange system. The exit of protons, secondary to growth factor action, initiates the cascade of cationic movement across the membrane: increased Na^+ influx and Na^+/K^+ pump activation (8). Therefore, activation of these ionic movements is common to all cell systems, as is common activation of glycolysis and other metabolic pathways linked to onset of protein and nucleic acid synthesis. In contrast, in model 2, the interaction of growth factors with their specific membrane receptors directly activates the Na^+/H^+ exchange system, leading to an increase in intracellular pH as one of the first signals. Note that other signals parallel to proton release also take place; amiloride only slightly inhibits phosphorylation of the 27,000-dalton peptide (Fig. 5 *A* and *B*). Preliminary data are in favor of model 2. As expected from this model, we found that growth factors enhance the Na^+/H^+ exchange system independently of cell metabolism activation.

One of the most important features shared by the two models consists in the coupling of S6 phosphorylation to proton release. Ribosomal protein S6 is not, or is poorly, phosphorylated in quiescent cells; its rapid phosphorylation on addition of serum, purified growth factor, insulin in various cell types (refs. 9, 25–27; unpublished), or sperm in sea urchin eggs (28) precedes stimulation of protein synthesis. Although no precise role has yet been assigned to the phosphorylated form of S6, its phosphorylation involving cAMP-independent protein kinase (27) seems a prerequisite to protein synthesis activation in arrested cells. One of the earliest indications of the important role of intracellular pH in cell "activation" came from studies of sea urchin eggs. Thus, the pH of these eggs increased within minutes after fertilization (7, 29), suggesting that the lower pH of unfertilized eggs keeps protein synthesis turned off and that it is turned on by the pH increase that follows sperm penetration or exposure to weak bases (29). This report showing that growth factor-stimulated S6 phosphorylation is under the control of intracellular pH is of particular importance. This conclusion is based on the fact that two types of drugs (protonophores and amiloride) that lower intracellular pH prevent S6 phosphorylation. Other examples along this line exist: Moore (21) has shown a Na^+-dependent elevation of internal pH in response to insulin in frog muscle, and Horne *et al.* recently reported a thrombin-induced pH change during platelet aggregation (30). Therefore, we speculate that a crucial role of various effectors including growth factors is to increase intracellular pH via activation of a common Na^+/H^+ exchange system. Even though no direct proof exists, this signal may play an important role in induction of a variety of biological events (e.g., cell division, secretion, aggregation, neurite extension) depending on the target cell studied.

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