Extracellular Na^+ and Initiation of DNA Synthesis: Role of Intracellular pH and K^+

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ABSTRACT Initiation of DNA synthesis in confluent quiescent 3T3 cell cultures stimulated by epidermal growth factor (EGF), vasopressin, and insulin was abolished by removing extracellular Na^+. The inhibition was reversible, time- and Na^+-concentration-dependent, and not due to an effect on binding or internalization of ^{125}I-EGF. Stimulation by combinations of other growth factors with diffrent mechanisms of action was also affected by decreasing extracellular Na^+, but with different half-maximal Na^+ concentrations. When choline was used as an osmotic substitute for Na^+, the decrease in DNA synthesis was correlated with the decrease in intracellular K^+. In contrast, when sucrose was used there was stimulation of the Na^+-K^+ pump and maintenance of intracellular K^+ that resulted in a somewhat higher rate of DNA synthesis at lowered extracellular Na^+ compared to choline. Mitogenesis induced by epidermal growth factor, vasopressin, and insulin led to cytoplasmic alkalinization as determined by an increase in uptake of the weak acid 5,5-dimethyloxazolidine-2,4-dione. Experimental decrease in extracellular Na^+ blocked this cellular alkalinization. Therefore, under some conditions the supply of extracellular Na^+ may limit cellular proliferation because of a reduction in the provision of Na^+ to the Na^+/H^+ antiport and resultant failure of alkalinization. We conclude that Na^+ flux and its effect on intracellular K and pH has a major role in the complex system that regulates proliferation.

Quiescent cultures of 3T3 cells initiate DNA synthesis and cell division when fresh serum or growth factors are added to the media (26). Changes in the transmembrane flux of monovalent ions have been implicated in the regulation of this proliferative response (27, 29). A rapid increase in Na^+ entry stimulates activity of the Na^+/K^+ pump (33, 39) and increases intracellular K^+ (33, 41) that is associated with commencement of DNA synthesis (10). Na^+ influx is also coupled with proton exit by an electroneutral, amiloride-sensitive Na^+/H^+ antiport that modulates intracellular pH (38). However, those mitogens that increase cell cAMP stimulate the Na^+/K^+ pump by a mechanism other than Na^+ influx (22). Therefore, Na^+ entry is part of a complicated system through which it can affect intracellular K^+ and pH. It may be linked to intracellular ionic events that themselves represent a convergence point for initiation and control of mitogenesis stimulated by diverse agents.

The hypothesis that Na^+ influx plays an important role in mitogenesis depends upon three lines of evidence. First, that serum or mitogenic factors added in serum-free medium stimulate Na^+ influx (4, 14, 15, 32, 39) and H^+ efflux (38). Ionic fluxes have been widely documented in 3T3 cells (14, 15, 32, 39, 40) and subsequently in many other cell types, including human fibroblasts (14, 18, 20, 42), hamster fibroblasts (14, 21), rat liver cells (8), neuroblastoma cells (17), lymphocytes (6), and BSC-1 epithelial cells (25). Second, the induction of ion fluxes acts in synergy with other events to induce mitogenesis. For example, the membrane permeability modulators mellitin (32) and amphotericin B (28) as well as peptides that induce ion fluxes in their target cells such as vasopressin (34) and bombesin (37) stimulate quiescent 3T3 cells to enter S-phase. Third, a decrease in the rate of influx of Na^+ prevents the development of the mitogenic response. For example, a reduction of Na^+ in the culture medium slows S-phase entry in 3T3 cells stimulated by serum (39) or by epidermal growth factor (EGF)[1] and insulin (27).

Several recent findings complicate the interpretation of the

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; DMO, 5,5-dimethyl 2-^{14}C oxazolidine-2,4-dione; EGF, epidermal growth factor; IBMX, 3-isobutyl-1-methylxanthine; PDGF, platelet-derived growth factor.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MARCH 1984 1082-1089

cause and effect relationship between mitogen-induced Na^+ fluxes and the elicitation of the proliferative response. Balk and Polimeni (1) and Lubin (12) reported that the effect of reduction of extracellular Na^+ on mitogenesis depends on the Na^+ substitutes used for osmotic stabilization. Furthermore, the fact that an inhibitor of Na^+—H^+ exchange, amiloride, decreases the mitogenic response (8, 18, 36) may be due to an effect of the drug on protein synthesis (9, 13) rather than a specific effect on Na^+ ion movement.

The causality between Na^+ fluxes and the stimulation of DNA synthesis is of crucial importance for the hypothesis that implicates ion fluxes in the control of cell proliferation. Since this aspect of the hypothesis remains controversial, we decided to re-examine the relationship between early ionic events and the initiation of DNA synthesis promoted in serum-free medium by a variety of defined mitogenic molecules. Our experiments were designed to study the effect of blocking mitogenesis in serum free media by experimentally varying extracellular Na^+. The resultant observed relationships between mitogenesis and intracellular K^+ and pH offer further evidence that Na^+ entry and the ionic events that it stimulates, are central in the reinitiation of DNA synthesis by quiescent 3T3 cells.

MATERIALS AND METHODS

Cell Culture: Swiss 3T3 cell stock cultures were grown at 37°C in humidified 10% CO_2/90% air in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). For experiments, 10^5 cells were subcultured into 30-mm Nunc petri dishes with media containing 10% fetal bovine serum. Such subcultures were used 6–8 d later at a time when the cells were confluent and quiescent as judged by cytofluorometric and autoradiographic analysis (33).

Preparation of Low-Na^+ Media: Media containing variable concentrations of Na^+ were prepared by adding NaCl to modified DME-Waymouth Medium (1:1, vol:vol) (16) containing Hepes-Tris buffer (20 or 38 mM) and <10 mM Na^+. Media were made isotonic by adding choline chloride to a total osmotic equivalent of 110 mM. Alternatively sucrose was used for osmotic replacement at twice the concentration of choline since it provides only one osmotically active molecule. The Na^+ of all media was verified by flame photomtery.

DNA Synthesis and Autoradiography: Cells were washed four times with DME containing 110 mM choline chloride, 20 mM HEPES-Tris buffer and <10 mM Na^+. Incubations were carried out in 2 ml DME-Waymouth medium with 20 mM HEPES-Tris buffer and [^{3}H]thymidine (0.94 μM, 1.06 Ci/mmol) before determination of radioactivity incorporated into trichloroacetic acid precipitable material (33). Autoradiography was performed as described (33).

Intracellular Electrolyte Content and ^{86}Rb Uptake: Cultures were washed five times with 0.1 M $MgCl_2$ at 4°C. After the dishes were dry, 0.4 ml of 5% trichloroacetic acid, containing 15 mM LiCl as an internal standard, was added to lyse the cells. Na^+ and K^+ were measured with a Corning EEL, Model 30 flame photometer (Evans Electoselenium Ltd., Essex, England). For Na^+ uptake measurements, the accumulation of Na^+ in the cells was measured when the active efflux of this cation via the Na-K pump was blocked using 2 mM ouabain. ^{86}Rb uptake was measured as previously described (22, 40).

Intracellular pH: Intracellular pH was estimated using the weak acid 5,5-dimethyl [2-^{14}C]oxazolidine-2,4-dione (DMO) (23, 43) using a modification of a previously described method (38). Briefly, cultures were washed three times with modified DME containing 110 mM choline chloride, <10 mM NaCl and 38 mM HEPES-Tris buffer (pH 7.0–7.1). Modified DME-Waymouth medium (0.9 ml) containing 38 mM HEPES-Tris and various concentrations of Na^+ was added and cultures incubated at 37°C for 1 h before the addition of [^{14}C]DMO (final concentration in dish 150 μM; 1.4–1.8 × 10^6 cpm/dish). After 15 min further incubation, the cells were washed three times with 0.1 M $MgCl_2$ at 4°C. This rapid washing that was completed in <5.5 s has been shown to result in only 10–15% loss of intracellular DMO (38). The monolayers were then dried and the cells solubilized with 0.1 M NaOH/2% Na_2CO_3/0.1% SDS, and radioactivity was measured using a liquid scintillation spectrometer.

Intracellular pH was calculated from extracellular pH, [^{14}C]DMO in washed monolayer and media, protein (11) and intracellular water (see below) using the formula of Waddell and Butler (43). Measurement of extracellular water was obviated by rapid washing with cold $MgCl_2$ three times before appreciable efflux of DMO (38). DMO trapped in extracellular water after these washes was corrected for by subtracting the radioactivity remaining in pre-cooled cells washed immediately after addition of [^{14}C]DMO. Four replicates were determined for each experimental value. A pKa of 6.3 was used for calculations.

Intracellular Water: Intracellular water was determined using [^{14}C]urea. Urea distributes into total water space and can therefore be used for measurement of intracellular water space if that amount distributed in extracellular fluid can be accounted for or removed by washing. Extracellular [^{14}C]urea was removed by five rapid washes (10 s) of the monolayer each with 5-ml aliquots of cold 0.1 M $MgCl_2$. During the 10 s required for five rapid washes, only negligible amounts of urea leaked out of the monolayer. At 4°C there was little efflux of urea even after 2 min. In contrast, there was considerably more rapid efflux of the marker when the wash solution was at 37°C. For measurements of intracellular water, the cultures were incubated for 1 h in 0.96 ml of modified DME-Waymouth medium containing 20 mM HEPES-Tris buffer (pH 7.0) with or without EGF, vasopressin, and insulin and containing 109–112 mM or 7 mM Na^+. Then 40 μl of [^{14}C]urea were added (final 0.1 mM, 0.85–1.02 × 10^6 cpm/dish) and after 15-min cultures were washed rapidly five times with 0.1 M $MgCl_2$ at 4°C. Trapped [^{14}C]urea that was extracellular but not removed by the washing was estimated in each experiment by rapid washing of pre-cooled dishes immediately after the addition of [^{14}C] urea. These values were subtracted from total counts of the monolayer to obtain intracellular cpm.

^{125}I-EGF Binding: These studies were performed as described (31). After incubation with ^{125}I-EGF cells were washed four times with cold phosphate-buffered saline containing 0.1% bovine bovine serum albumin and 0.1 μM potassium iodide. They were then extracted with 0.2 M acetic acid/0.15 M NaCl at 4°C for 6 min to obtain surface bound EGF (7) and with 0.1 M NaOH/2% Na_2CO_3/2% SDS for internalized ^{125}I-EGF.

Materials: Crystalline bovine insulin, cholera toxin, ouabain, and arginine vasopressin were obtained from Sigma Chemical Co. (St. Louis, MO), fetal bovine serum from Gibco Europe (Paysley, Scotland, U.K.) and 3-isobutyl-1-methyl-xanthine (IBMX) from Aldrich Chemical Co. (Dorset, England). EGF was obtained from Collaborative Research Inc. (Waltham, MA). Human platelet-derived growth factor (PDGF) was partially purified as described by Deuel et al. (3), and was a gift from Dr. P. Stroobant. [^{3}H]Thymidine was purchased from the Radiochemical Centre (Amersham, England) and [^{14}C]urea and [2-^{14}C]DMO were from New England Nuclear (Boston, MA). ^{125}I-EGF (1.44 × 10^5 cpm/ng) was prepared using Na^{125}I (Radiochemical Centre, Amersham, England) and receptor grade EGF (Collaborative Research Inc., Waltham, MA) (31). Amiloride (*N*-amidino-3,5-diamino-6-chlorpyrazinecarboxamide) was a gift from Merck, Sharp and Dohme (Hertfordshire, England). All other materials were reagent grade.

RESULTS

Effect of Extracellular Na^+ on Mitogenesis and Na^+ Flux

DEPENDENCE OF S-PHASE ENTRY STIMULATED BY EGF, VASOPRESSIN, AND INSULIN ON EXTRACELLULAR Na^+: To determine the effect of the concentration of Na^+ in the culture medium on the stimulation of DNA synthesis by Swiss 3T3 cells, we incubated quiescent cultures of these cells with EGF, vasopressin, and insulin in serum-free media of variable Na^+ concentration and containing [^{3}H]thymidine. The incorporation of radioactivity into trichloracetic acid–precipitable material was measured after a 26 h incubation. As shown in Fig. 1, there was a striking dependence of thymidine incorporation stimulated by EGF, vasopressin, and insulin on the Na^+ concentration of the medium. The incorporation was more than 100-fold less by cells in media containing 13 mM Na^+ as compared to cells in 120 mM Na^+. The relationship was approximately sigmoidal with a plateau at 70 mM and with half maximal point of 35 mM Na^+. The level of thymidine incorporation of cultures incubated in media with 13 mM Na^+ was similar to that of quiescent cells (10 vs. 18 cpm/μg protein). In the absence of vasopressin, DNA synthesis stimulated by EGF and insulin

was dependent on medium Na^+ as with the three peptides, but the curve was shifted to the right. The results were the same when thymidine incorporation was expressed as cpm per culture (data not shown). Furthermore, the relationship was virtually identical when DNA synthesis was measured by the percentage of labeled nuclei (Fig. 1). Thus, removal of Na^+ from the medium block the stimulation of DNA synthesis induced by peptide growth factors in serum-free medium.

REVERSIBILITY OF INHIBITION BY LOW Na^+: We examined whether the inhibition of mitogenesis brought about by low Na^+ concentration in the media is reversible or whether it induces a toxic effect which cannot be overcome by restoring Na^+. As can be seen in Table I, those cultures incubated with 17 mM Na^+ in which the extracellular Na^+ was raised to 115 mM after 26 h incorporated [^{3}H]thymidine at the same rate as the cultures that were kept in the presence of 115 mM Na for the entire 52 h. Thus, the inhibitory effect of Na^+ removal is fully reversible.

KINETICS: We compared the time course of cumulative [^{3}H]thymidine incorporation into 3T3 cells stimulated with EGF, vasopressin, and insulin in media containing 30 mM Na^+ vs. 115 mM (Fig. 2). The initiation of entry into S-phase appeared to commence at approximately the same time. However, the final maximum level and rate of entry into mitogenesis were greater for the cultures in 115 mM Na^+. Although the plateau of thymidine incorporation occurred at ~40 h, many of our studies were terminated at 26 h since it has been shown that after 30–40 h there is a time dependent ionic adaptation in 3T3 cells that are incubated in low K^+ (10).

Na^+ FLUX: As shown in Fig. 3, addition of EGF, vasopression, and insulin caused a marked increase in the rate of Na^+ uptake into 3T3 cells, a result in line with previous studies (15). We have now found that the rate of Na^+ uptake by cultures stimulated with peptide factors becomes similar to that of quiescent cells when the Na^+ concentration in the

TABLE I

Reversibility of the Inhibition of Mitogenesis by Low Extracellular Na^+

Extracellular Na^+		Thymidine incorporation	Intracellular K^+
0–26 h	26–52 h		
mM		*cpm/μg protein*	*μmol/mg protein*
17	17	97	0.39
115	115	1,514	1.09
17	115	1,617	1.06

3T3 cultures were incubated for 26 h in serum-free DME-Waymouth medium with EGF, vasopressin, and insulin and containing 17 or 115 mM Na^+ replaced in equiosmotic amounts with choline chloride. Fresh medium containing either 17 or 115 mM Na^+ was then added. [^{3}H]Thymidine added to the cultures during the last 26 h was used to estimate DNA synthesis. Intracellular K^+ was determined by flame photometry. Values are mean of two similar values.

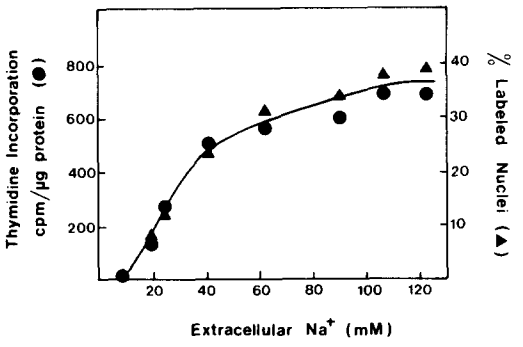

FIGURE 1 DNA Synthesis by 3T3 cells as a function of Na^+ concentration in the culture medium. Cultures of quiescent 3T3 cells were washed four times with Na^+-free DME-Waymouth medium and incubated for 26 h with vasopressin (20 ng/ml), EGF (5 ng/ml), and insulin (1 μg/ml) in fresh serum-free media containing different concentrations of Na^+ and [^{3}H]thymidine. DNA synthesis was measured by incorporation of [^{3}H]thymidine into trichloroacetic acid precipitable material (circles) or by autoradiography (triangles). Cells in medium containing no factors (full Na^+) gave 1% labeled nuclei, and cells exposed to 10% fetal bovine serum had 59% labeling. Na^+ was measured by flame photometry. Shown are the mean of three to five replicate cultures in a representative experiment.

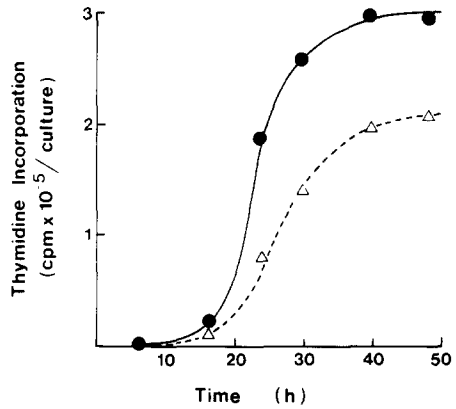

FIGURE 2 Time course of S-phase entry of 3T3 cells stimulated by EGF, vasopressin, and insulin in media of various Na^+ concentrations. Cultures were incubated in media containing 115 mM Na (circles) or 30 mM Na replaced with choline chloride (triangles).

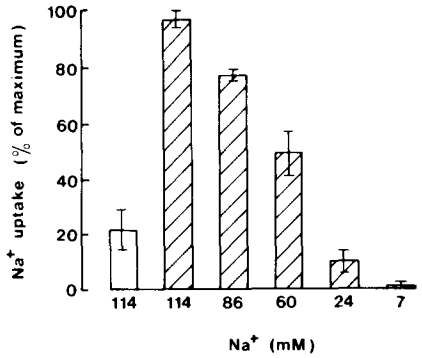

FIGURE 3 Relationship of Na^+ uptake to extracellular Na^+. Cultures of 3T3 cells were incubated in DME-Waymouth medium containing 20 mM HEPES-Tris buffer (pH 7.2) and various concentrations of Na^+. Except for quiescent nonstimulated cultures, medium contained EGF, vasopressin, and insulin. Choline chloride was used for osmotic stabilization in the low Na^+ medium. After incubation for 1 h, ouabain (2 mM) was added 30 min before five washes with 0.1 M $MgCl_2$ at 4°C. The Na^+ contents of parallel cultures not exposed to ouabain were also measured. The values were subtracted from the Na^+ concentrations measured in the presence of ouabain, so the values represent net increase of total cell Na^+. The cell cultures were dried and disrupted with 0.4 ml 5% trichloroacetic acid containing 15 mM lithium chloride as internal standard. Na^+ was measured on a flame photometer. Shown are the results of three separate experiments normalized to 100% and combined. The 100% value was 104 ± 3 nmol/mg protein. Each bar is the mean ± S.E. of at least nine cultures. Empty bar, no additions; striped bar, EGF, vasopressin, and insulin.

medium is reduced from 114 mM to ~25 mM (Fig. 3). Inspection of Fig. 1 reveals that at this concentration of Na^+ in the medium, the mitogenic response to the peptide factors is reduced by 60%. Thus, adjusting the rate of Na^+ flux in stimulated cells to that of quiescent cells by reducing the Na^+ concentration in the medium markedly reduces mitogenesis and provides evidence for a causal relationship of cell Na^+ movements and initiation proliferation.

Ionic Implications of Growth Factor-stimulated Na^+ Entry

Changes in Na^+ influx can influence intracellular K^+ since Na^+ exit is obligatorily linked to K^+ entry via the Na-K pump. Further, an important pathway of Na^+ entry in mitogen-stimulated 3T3 cells is coupled to H^+ exit through an electroneutral, amiloride-sensitive Na^+/H^+ antiport. Thus, the dependence of DNA synthesis on Na^+ can result, at least partly, from alterations in intracellular K^+ and/or changes in intracellular pH. In an effort to clarify the mechanism whereby stimulation of DNA synthesis depends on Na^+, we studied the role of intracellular K^+ and pH.

RELATIONSHIP OF MITOGENESIS AND INTRACELLULAR K^+: Quiescent Swiss 3T3 cells were stimulated to enter DNA synthesis by EGF, vasopressin, and insulin in the presence of variable concentrations of extracellular Na^+ and the incorporation of [^{3}H]thymidine and the level of cell K^+ were measured after a 26-h incubation. As shown in Fig. 4, the plot of thymidine incorporation as a function of intracellular K^+ is steeply sigmoidal. Interestingly, a similar relationship was obtained for cells stimulated with EGF and insulin and the plot was identical to the lower portion of the curve obtained from cells stimulated with three factors (Fig. 4, triangles). The fact that the relationship of mitogenesis and cell K^+ is identical for stimulation with combinations of mitogenic factors of two degrees of potency indicates a close relationship between cell K^+ and DNA synthesis. Furthermore, this correlation is maintained in experiments in which the effect of low concentrations of extracellular Na^+ are reversed (Table I).

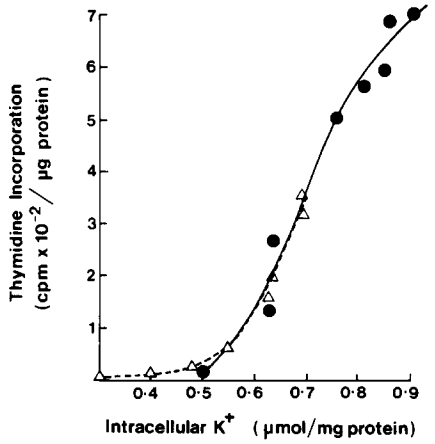

FIGURE 4 Relationship of DNA synthesis to intracellular K^+ achieved by varying the extracellular Na^+. Cultures were incubated with EGF, vasopressin, and insulin (circles) or EGF and insulin (triangles) in serum-free DME-Waymouth medium containing variable concentrations of Na^+. Choline chloride was used as an osmotic replacement for Na^+. After 26 h, cells that had been washed five times with cold 0.1 M $MgCl_2$ were disrupted with 5% trichloroacetic acid containing 15 mM lithium chloride and K^+ was determined by flame photometry. DNA synthesis was estimated by [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material.

SUCROSE AS AN OSMOTIC SUBSTITUTE AND THE ROLE OF CELL K^+: In the previous experiments choline chloride was used as an osmotic stabilizer to replace Na^+. Disaccharides that are excluded by cells because of their size can also be used, and we chose sucrose for comparison to choline chloride. When thymidine incorporation stimulated by EGF and insulin was examined as a function of Na^+ in the medium, there was dependence of DNA synthesis on extracellular Na^+. However compared to the studies using choline chloride, this relationship was different (Fig. 5). Osmotic replacement with sucrose shifted the curve to the left and reduced the half maximal point from ~75 to 37 mM.

Interestingly, for all extracellular Na^+ concentrations, intracellular K^+ was higher when sucrose was used as osmotic replacement as compared to choline (Fig. 6). This demonstrates that 3T3 cells maintain K^+ within the cell in the presence of sucrose even at lowered medium Na^+ concentration and this cell K^+ level is associated with a higher rate of DNA synthesis (Fig. 5).

Na-K PUMP ACTIVITY: The higher concentration of intracellular K^+ associated with the use of sucrose as an osmotic substitute for Na^+ could result from either increased activity of Na^+-K^+ pump or, alternatively, to decreased rate of K^+ efflux from the cell. Uptake of the K^+ tracer, ^{86}Rb, was

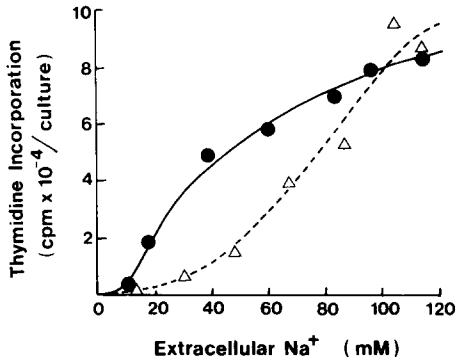

FIGURE 5 Effect of sucrose versus choline as osmotic substitutes for Na^+. Cultures were exposed to EGF and insulin for 26 h in DME-Waymouth medium containing variable concentrations of Na^+. DNA synthesis was estimated by [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material. Either sucrose (circles) or choline (triangles) was used as an osmotic replacement of Na^+.

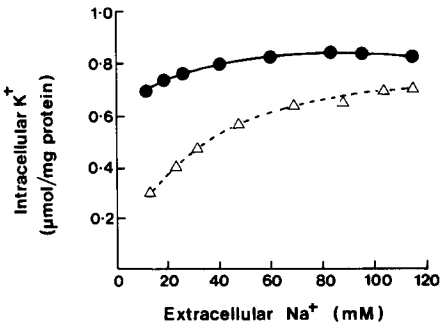

FIGURE 6 Effect of choline chloride and sucrose as osmotic stabilizers on intracellular K^+. Cultures stimulated with EGF and insulin for 26 h were harvested and analyzed for cellular K^+. Sucrose (circles) and choline chloride (triangles).

used to measure Na^+-K^+ pump activity. We compared the effect of choline or sucrose used as osmotic substitute in medium containing only 27 mM Na^+ (Table II). In the presence of mitogenic factors, there was a 43% greater ^{86}Rb uptake with sucrose as compared to choline. Table II also shows that in this Na^+ depleted medium the growth factors stimulated pump activity only when sucrose was used.

The increased activity of the pump in medium with low Na^+ replaced with sucrose was not due to differences in Na^+ influx. Sucrose as compared to choline had no effect on Na^+ uptake (0.137 ± 0.011 vs. 0.145 ± 0.049 μmol/30 min/mg protein) or amiloride-sensitive Na^+ uptake (0.048 ± 0.011 vs. 0.062 ± 0.014 μmol/30 min/mg) of 3T3 cells stimulated with 10% dialyzed fetal bovine serum.

On the basis of this portion of the study, we concluded that the use of sucrose to maintain osmotic pressure in medium low in Na^+ is associated with increased activity of the Na^+-K^+ pump that maintains the steady state level of cell K^+ even in medium containing low concentrations of Na^+. This increase in Na^+-K^+ pump and intracellular K^+ may explain the greater mitogenic effect of growth factors in medium containing sucrose to replace Na^+.

RELATIONSHIP OF DMO UPTAKE TO Na^+ AND MITOGENESIS: The use of sucrose as a Na^+ substitute provides a striking example of dependence of DNA synthesis on Na^+ concentration in the medium without decrease in cellular K^+. This result emphasizes that under certain experimental conditions the influence of Na^+ on DNA synthesis can be the result of other processes. In view of the existence of a mitogen-sensitive Na^+/H^+ antiport in 3T3 cells (38), we studied the effect of EGF, vasopressin, and insulin on intracellular pH and its dependence on Na^+ entry.

Since Na^+ exchanges for H^+ through an amiloride-sensitive antiport, we examined the effect of extracellular Na^+ on intracellular pH. Fig 7 shows that DMO uptake decreases as Na^+ is removed from the medium. In nine total experiments the DMO uptake of stimulated cells in medium with 8 mM extracellular Na^+ was 431 ± 40 cpm/culture compared to 653 ± 38 cpm/culture when the media contained 117 mM Na^+ ($P < 0.001$). In a representative experiment, DMO uptake of cultures incubated in medium containing 7 mM Na^+ was only 61% that of cultures incubated in 114 Na^+ (Fig. 8*A*). Furthermore, the DMO uptake of these cultures in low extracellular Na^+ was similar in magnitude to that of the quiescent cultures. Fig. 8*B* shows Na^+ flux under the same conditions for comparison. Na^+ entry into cultures in medium with low Na^+ was decreased as was the Na^+ entry of nonstimulated cells. This pattern of decrease in Na^+ influx in both quiescent

TABLE II

Effect of Osmotic Substitute for Na^+ on Ouabain-inhibited ^{86}Rb Uptake

	Mitogens	No mitogens	*P*
	nmol/mg/min		
Choline	3.20 ± 0.20	2.50 ± 0.45	NS
Sucrose	4.59 ± 0.44	2.99 ± 0.27	<0.025
P	<0.05	NS	

Quiescent cultures were incubated for 15 min in DME-Waymouth medium containing 27 mM Na^+, 20 mM HEPES-Tris buffer (pH 7.15) and either choline chloride or sucrose for osmotic stabilization. Cultures with mitogens contained EGF (5 ng/ml), vasopressin (20 ng/ml), or insulin (1 μg/ml). Ouabain (2 mM) was added to half the dishes 10 min before ^{86}Rb (2.1×10^6 cpm/culture). After 20 min, cultures were washed five times with cold 0.1 M $MgCl_2$, 1 ml of 5% trichloroacetic acid was added and radioactivity assayed in a liquid scintillation counter. Ouabain-inhibited ^{86}Rb uptake was calculated as the difference between uptake in the absence and presence of inhibitor. Shown are mean and SE of dishes from two separate experiments. ^{86}Rb uptake by cultures in 122 mM Na^+ were 5.77 nmol/mg/min (mitogens) and 3.58 nmol/mg/min (no mitogens). *NS*, not significant.

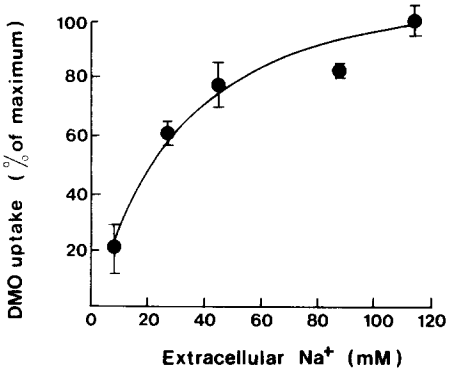

FIGURE 7 Relationship of DMO uptake and extracellular Na^+. Modified DME-Waymouth medium with 38 mM HEPES-Tris buffer and containing EGF, vasopressin, insulin, and various concentrations of Na^+ was added to 3T3 cultures. Osmotic replacement was accomplished using choline chloride. After 1-h incubation at 37°C in 10% CO_2/90% air, [^{14}C]DMO was added (final concentration 150 μM, 1×10^6 cpm/dish) for 15 min. The cultures were then washed three times with 0.1 M $MgCl_2$ at 4°C. This washing was accomplished in <5.5 s. Cells were solubilized and radioactivity determined. The 100% value was 809 ± 43 net cpm/culture (mean ± SE; $n = 4$).

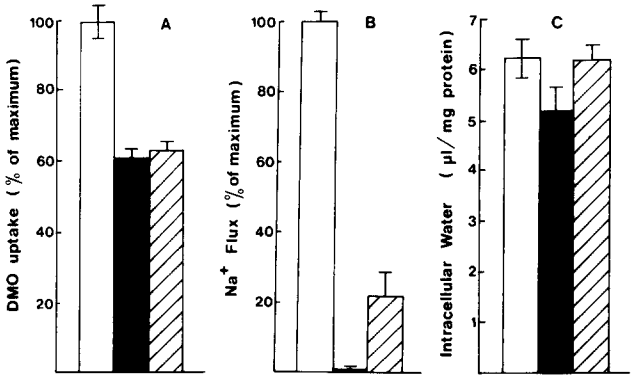

FIGURE 8 Relationship of extracellular Na^+ to DMO uptake, Na^+ influx, and intracellular water. DME-Waymouth medium containing various concentrations of Na^+ and 20 mM HEPES-Tris buffer (pH 7.2) was added to cultures that had previously been washed three to four times with Na^+ free DME. Except for quiescent nonstimulated cultures, medium contained EGF, vasopressin, and insulin. Choline chloride was used for osmotic stabilization in the low Na^+ media that contained 7 mM Na^+. (*A*) DMO uptake used to estimate intracellular pH is shown. Methods as in Fig. 7. The 100% value (i.e. that of cultures incubated with EGF, vasopressin, and insulin at 114 mM Na^+) was 1,117 ± 58 cpm/culture (mean ± S.E.; $n = 4$). The DMO uptake in the absence of factors (extracellular Na^+114 mM) and in low Na media are significantly lower than in the presence of factors ($P < 0.005$). (*B*) Na^+ flux—after incubation for 1 h, ouabain (2 mM) was added 30 min before five washes with 0.1 M $MgCl_2$ at 4°C. The cell cultures were dried and disrupted with 0.4 ml 5% trichloroacetic acid containing 15 mM lithium chloride as internal standard. Na^+ was measured on a flame photometer. Shown are the results of three separate experiments normalized to 100% and combined. The 100% value was 104 ± 3 nmol/mg protein. Each point is the mean of nine dishes. (*C*) Intracellular water was determined on the same set of cultures as *A*. The 100% value was 6.35 ± 0.39 μl/mg protein (mean ± SE; $n = 4$). None of the values are statistically different. Empty bars, 114 mM Na^+; filled bar, 7 mM Na^+; striped bar, quiescent cells.

cells and cells stimulated with growth factors in low medium Na^+ is much like that of DMO uptake. Taken together these data provide evidence for a role of cytoplasmic alkalinization via the Na^+/H^+ antiport in growth factor function. Therefore, prevention of cytoplasmic alkalinization could be an additional mechanism by which depletion of Na^+ in the medium inhibits mitogenesis induced by growth factors.

Schuldiner and Rozengurt (38) have shown that Swiss 3T3 cells stimulated to enter S-phase with PDGF, vasopressin, and insulin have a higher intracellular uptake of DMO and higher pH than unstimulated cells. In the present studies cells stimulated with EGF, vasopressin, and insulin accumulated a higher amount of DMO (653 ± 38 cpm/culture, $n = 34$) as compared to unstimulated cells (484 ± 46 cpm/culture, $n = 28$, $P < 0.01$) in nine different experiments.

That changes noted in DMO uptake are not simply due to a change in intracellular water is indicated by the results shown in Fig. 8*C*. Intracellular water of our cells measured with urea was 5.94 ± 0.26 μl/mg protein ($n = 12$). Neither the presence of the growth factors EGF, vasopressin, and insulin nor changes in extracellular Na^+ had an appreciable effect on intracellular water. The calculated values of intracellular pH were 7.09 ± 0.03 for cells stimulated with EGF, vasopressin, and insulin vs. 6.88 ± 0.04 for quiescent cells. This confirms the previous observation that mitogenesis is accompanied by an increase in cellular pH (38).

EFFECTS OF EXTRACELLULAR Na^+ ON ^{125}I-EGF BINDING: Dickson et al. (5) have reported that extracellular Na^+ is necessary for maximal receptor mediated endocytosis of several ligands including EGF. To determine the effect of extracellular Na^+ on the interaction of EGF with 3T3 cells, exposed cultures to different concentrations of ^{125}I-EGF either at 24 mM or 120 mM Na^+ in the medium. After 1 h of incubation, the cultures were washed and the cell-associated radioactivity was separated into surface-localized and internalized by the acid treatment described by Haigler et al. (7). In agreement with the observations of Dickson et al. (5) we saw a decline in ^{125}I-EGF internalization at low Na^+ concentrations (Fig. 9). At an EGF concentration of 5 ng/ml, which was used in the DNA synthesis assays, there was only a small but significant difference ($P < 0.05$). It is unlikely that this small effect of lowered extracellular Na^+ on EGF internalization explains its effect on mitogenesis and ionic fluxes for the following reasons. First, at an extracellular Na^+ concentration of 8 mM, total cell associated ^{125}I-EGF at 5 ng/ml was decreased by only 20% (Table III) while DNA synthesis was

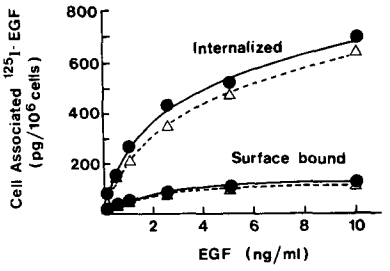

FIGURE 9 Effect of extracellular Na^+ on ^{125}I-EGF binding. Quiescent cultures of 3T3 cells were washed twice and incubated at 37°C in binding medium containing ^{125}I-EGF and 127 mM Na^+ (circles) or 24 mM Na^+ (triangles). The later medium was made isotonic by addition of choline Cl. After incubation for 1 h, the cells were washed and the surface bound and internalized radioactivity was determined.

TABLE III

Total Cell-associated ^{125}I-EGF

EGF	Extracellular Na^+ 8 mM	Extracellular Na^+ 120 mM
	pg/10^6 cells	
5	446 ± 17	558 ± 28
10	725 ± 26	766 ± 16

Quiescent cultures of 3T3 cells were washed and incubated in medium containing ^{125}I-EGF at the designated EGF concentrations. Choline was added to the 8 mM Na^+ medium to make it isotonic. After incubation for 1 h at 37°C, the cells were washed and total cell associated radioactivity determined. Shown are mean ± S.E. of four determinations.

<1% (Fig. 1). Second, if the small decrease of cell-associated ^{125}I-EGF at 5 ng/ml in low Na^+ medium were physiologically significant, then 10 ng/ml of EGF should abolish the inhibitory effect of low Na^+ on thymidine incorporation, since the total cell-associated ^{125}I-EGF at 10 ng/ml EGF in low Na^+ was greater than at 5 ng/ml EGF in full Na^+. In fact, the effect of Na^+ on DNA synthesis persisted with double the concentrations of EGF (data not shown). Therefore, we conclude that the effect of medium Na^+ on DNA synthesis and ionic fluxes is not due to an effect on EGF internalization.

Na^+ DEPENDENCE WITH OTHER FACTOR COMBINATIONS: PDGF, one of the most potent mitogens for 3T3 cells (24) is known to stimulate Na^+ entry (14), therefore it was anticipated that like EGF and vasopressin there would be dramatic dependence of mitogenesis on extracellular Na^+. Fig. 10 shows that at 25 mM Na^+ thymidine incorporation was 31% of maximum (mean 26 ± 9 in four experiments) and the half maximal point was 33 mM.

Recently, increased levels of cAMP were demonstrated to act synergistically with other growth promoting factors to stimulate DNA synthesis (30, 35). Since increased levels do not stimulate Na^+ entry into quiescent 3T3 cells (22), it appears that cAMP delivers a mitogenic signal through a mechanism not involving a primary increase in Na^+ dependent ion fluxes. Interestingly, the dependence of DNA synthesis on Na^+ concentration in the medium in cultures stimulated by cholera toxin, IBMX, and insulin is markedly shifted to the left (Fig. 10); at 25 mM Na^+ >70% of the maximal DNA synthesis persisted (mean of six experiments 72.3% ± 7.3) and the half maximal point was 20 mM, which is in marked contrast to the value obtained in cultures stimulated by EGF, vasopressin, and insulin, i.e., 35 mM (Fig. 10).

Stimulation of DNA synthesis in quiescent 3T3 cells was also dependent on the Na^+ concentration in the nutrient medium when the cultures were stimulated by the following combinations: PDGF, vasopressin, and insulin; vasopressin and insulin; cholera toxin, IBMX, vasopressin, and insulin; PGE_2, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine, and insulin; and dialyzed fetal bovine serum (data not shown).

Our findings demonstrate that although Na^+ is required in the medium for the stimulation of a mitogenic response, there are marked differences in the half-maximal concentration of Na^+ required for DNA synthesis initiated by the various mitogenic ligands with different mechanisms of action.

DISCUSSION

The results presented in this paper demonstrate that stimulation of DNA synthesis by EGF, vasopressin, and insulin depends on the concentration of Na^+ in the medium. Since

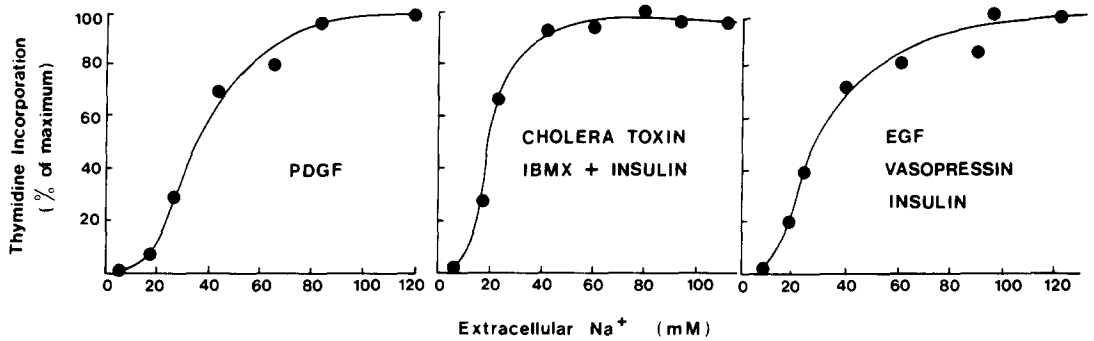

FIGURE 10 DNA synthesis as a function of Na^+ concentration in the culture medium. Cultures of quiescent 3T3 cells were washed four times with Na^+-free DME-Waymouth medium and incubated with *A*. PDGF (0.625 μg/ml); (*B*) cholera toxin (100 ng/ml), IBMX (50 μM), and insulin (1 μg/ml); (C) vasopressin (20 ng/ml), EGF (5 ng/ml) and insulin (1 μg/ml) in fresh serum-free media containing different concentrations of Na^+. The ionic strength was maintained by appropriate changes in choline Cl concentration. DNA synthesis was measured by incorporation of [^{3}H]thymidine into trichloroacetic acid precipitable material. Na^+ was measured by flame photometry.

adjusting the rate of Na^+ influx in stimulated cells to that seen in quiescent cells causes a 60% inhibition of the mitogen response, it seems that the increment in Na^+ influx is a necessary event in the mechanism whereby peptide growth factors stimulate initiation of DNA synthesis. Changes in Na^+ fluxes can influence the movement of other ions as well, namely K^+ and H^+. Indeed, the relationships of intracellular pH and K^+ to Na^+ fluxes and DNA synthesis demonstrated in our study can be envisaged as part of a complex integrated regulatory system. The mitogenic growth factors trigger a "Na^+ cycle" composed of Na^+ influx via an amiloride-sensitive Na^+/H^+ antiport which results in pH modulation, and Na^+ extrusion by Na-K pump that leads to K^+ accumulation. Activity of this cycle would couple Na^+/H^+ antiport and Na^+-K^+ pump activity and modulate intracellular pH and K^+ both of which may be proximate modulators of events leading to DNA synthesis. An important corollary that emerges from this hypothesis is that either intracellular pH or K^+ may control DNA synthesis under differing conditions.

In our studies of the effect of extracellular Na^+ on DNA synthesis, we noted a close relationship of cell K^+ and thymidine incorporation. The curve defining the relationship (Fig. 4) is similar in shape and half maximal point to that recently reported by Lopez-Rivas et al (10). In that publication the intracellular level of K was perturbed by experimentally changing the concentration of K^+ in the medium and left open the possibility that the decrease in thymidine incorporation could be due to an increase in internal Na^+ rather than a decrease in cell K^+ as the study proposed. The observation of our study, which shows the same relationship of DNA synthesis and cell K^+ when extracellular Na^+ is varied experimentally, confirms that intracellular K^+, at least in part, regulates S-phase entry in Swiss 3T3 cells stimulated by peptide factors.

Balk and Palimeni (1) and Lubin (12) have recently reported that the effect of reduction of culture medium Na^+ is dependent on what is substituted to maintain osmotic pressure. In our studies the relationship between extracellular Na^+ and DNA synthesis was maintained when both sucrose and choline chloride were used to maintain osmolarity. However, sucrose led to a higher rate of DNA synthesis for a given extracellular Na^+ concentration and a resultant decrease in the half maximal point of thymidine incorporation. Interestingly, we observed that this shift of the dose-response curve can be accounted for by the maintenance of a higher intracellular K^+ in cells when sucrose was used. When we replaced Na^+ with choline chloride, the extracellular Na^+ and intracellular K^+ were correlated. Furthermore, intracellular K^+ correlated with DNA synthesis. However, we found that when Na^+ was replaced by sucrose, intracellular K^+ remained high in low Na^+ medium even though DNA synthesis declined. This finding highlights the possibility that the influence of Na^+ on the initition of DNA synthesis when Na^+ was replaced by sucrose can be mediated by a parameter other than cell K^+.

Schuldiner and Rozengurt (38) have reported that 3T3 cells have a functioning Na^+/Na^+ antiport. Furthermore, mitogenic stimulation with PDGF, vasopressin, and insulin result in cytoplasmic alkalinization to the extent of 0.16 pH units (38). In our study the uptake of the weak acid DMO was less with lower medium Na^+ indicating a lower cytoplasmic pH. It seems likely that one major mechanism of regulation of mitogenesis by extracellular Na^+ is the extent of cytoplasmic alkalinization. Our study also shows that mitogenic stimulation leads to cytoplasmic alkalinization using a different combination of factors. We observed that stimulation of 3T3 cells with EGF, vasopressin, and insulin alkalinized intracellular pH by 0.21 units. Removing Na^+ from the medium lowered pH to the level of the quiescent cell. Since removal of Na^+ causes a marked decrease in intracellular pH it is plausible that this parameter may be a major rate limiting modulator in the presence of low extracellular Na^+ when sucrose is used as osmotic substitute (note similarity of the shape of the curves relating thymidine incorporation versus extracellular Na^+ replaced with sucrose (Fig. 5) and DMO uptake (Fig. 7). Conversely, when choline is used and intracellular K is not maintained (Fig. 6), the cell levels of that ion may limit DNA synthesis as extracellular Na^+ is decreased.

An alternative explanation for our findings is that Na^+ may affect the rate of binding of growth factors (5). This possibility has been tested by examining cell associated EGF. The small differences in rate of binding and internalization between cells in medium with low vs. full Na^+ are not sufficient to explain the differences in thymidine incorporation or ionic data.

To prove that a given early event in growth stimulation is causally related to the subsequent events leading to DNA replication is a very difficult task. However, mounting evidence indicates the ubiquity of Na^+ fluxes as an early event

in a variety of quiescent cells stimulated to proliferate (see introduction). In turn, the results presented here and in recent papers (10, 38) demonstrate that Na^+ fluxes affect both intracellular pH and K^+. These major ionic events may be involved in controlling important cellular processes linked to growth such as energy metabolism, cytoskeletal conformation, and macromolecular synthesis (2, 19). The complexity of the process emphasizes the fact that it is not adequate to simply examine the relationship of media Na^+ and DNA synthesis under conditions of one osmotic substitute and draw conclusions about the importance of the role of Na^+ influx or lack of it. Thus, our study contributes further evidence that ionic events such as Na^+ influx, increases in intracellular K^+ and cytoplasmic alkalinization may play a role in the molecular mechanisms regulating initiation of cell proliferation in this eucaryotic cell.

We thank James Sinnett-Smith for expert technical help in performing the ^{125}I-EGF binding studies.

C. P. Burns is recipient of a U. S. Public Health Service Career Development Award (CA 00324).

Received for publication 22 July 1983, and in revised form 31 October 1983.

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