Signalling mitogenesis in 3T3 cells: role of monovalent ion fluxes and cyclic nucleotides

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Quiescent 3T3 cells resting in the G0 phase of the cell cycle can be stimulated to reinitiate DNA synthesis by combinations of chemically diverse agents which act synergistically when added to cultures maintained in serum-free medium. Understanding of the mechanism(s) whereby extracellular agents interact in modulating cell proliferation requires the identification of the intracellular signals important for initiating a mitogenic response. Our recent evidence indicates that increases in ion fluxes involving Na+, H+, K+, and Ca2+ and elevations in cyclic AMP (cAMP) levels (produced by cholera toxin, adenosine agonists, prostaglandin E1, or cAMP derivatives) can act as mitogenic signals for Swiss 3T3 cells. We propose that initiation of DNA synthesis can be elicited by the synergistic interaction of two identifiable signals, namely, an enhanced rate of ion movements and an increase in the cellular level of cAMP.

Quiescent cultures of 3T3 cells, resting in the G0/G1 phase of the cell cycle, can be stimulated to recommence DNA synthesis and cell division by the addition of fresh serum or of combinations of growth-promoting factors which act in a synergistic fashion (1). Evidence from this laboratory supports the view that early changes in ion fluxes underlie the action of a variety of mitogens (1-4). Thus, growth-promoting agents including platelet-derived growth factor (5), vasopressin (2,6,7), phorbol esters (8), melittin (9), and fresh serum (10,11) cause a rapid increase in Na+ influx and Na/K pump activity in 3T3 cells. The enhanced influx of Na+ and/or the movement of other ions coupled to Na+ entry, such as H+ (12), could act as an early signal for initiating cell proliferation (1-4).

The possibility that cyclic nucleotides, cyclic AMP (cAMP) and cyclic GMP (cGMP), may regulate the proliferative response of quiescent fibroblastic cells has been the subject of a large and controversial literature (13). In 3T3 cells and other fibroblast cells, increased levels of cAMP are widely thought to reduce the rate of growth and inhibit the stimulation of DNA synthesis promoted by adding serum to quiescent cells (see ref. 14 for further references). An objection to many of these studies has been that these effects were elicited by high concentrations of analogues of cAMP and could be regarded as non-specific (14). Because these objections precluded
a definitive conclusion on the effects of cAMP on the initiation of DNA synthesis of 3T3 cells and because the stimulation of ion fluxes (as induced by vasopressin, phorbol esters, melittin) appears not sufficient to induce a proliferative response, we decided to evaluate further the effects of cAMP-elevating agents on the initiation of DNA synthesis of 3T3 cells. In contrast to previous reports, we found that increased cellular concentrations of cAMP act synergistically with growth-promoting agents to stimulate DNA synthesis in quiescent cultures of 3T3 cells.

**Materials and Methods**

Swiss 3T3 cells were maintained and subcultured as previously described (9,10). The assays of growth-promoting activity (10,17), ion fluxes and content (7-11), and cAMP levels (15) were conducted as described. The sources of hormones, cyclic nucleotide analogues, toxins, and drugs can be obtained from previous publications (5-12, 15-20).

**Results and Discussion**

Recently, we found that cholera toxin, at concentrations which increase cAMP levels in intact cells, promotes (rather than inhibits) the initiation of DNA synthesis in Swiss 3T3 cells stimulated by suboptimal levels of serum (14,15). Furthermore, cholera toxin added with insulin, vasopressin, or phorbol esters (15), or with the recently discovered tumour promoter teleocidin (16), synergistically stimulated DNA synthesis in cultures of Swiss 3T3 cells. As shown in Fig. 1, cholera toxin stimulated DNA synthesis in a concentration-dependent fashion in serum-free medium supplemented with insulin. The shape of the dose-response curves for inducing DNA synthesis and for increasing cAMP (15) was similar. Cholera toxin was as effective as epidermal growth factor in inducing DNA synthesis in 3T3 cells (Fig. 1).

The combination of cholera toxin and insulin stimulated DNA synthesis after a lag period of about 16 h (Fig. 2), which is similar to the lag period obtained after addition of serum or growth factors to cultures of 3T3 cells (6,9,17). The cellular content of cAMP was increased by cholera toxin after a lag period of 15 min, reached a maximum (7-fold increase) after 3 h of incubation, and remained elevated for at least 24 h (15).

If the primary effect of cholera toxin on the initiation of DNA synthesis by 3T3 cells is due to its activation of the adenylate cyclase and cellular accumulation of cAMP, inhibitors of cyclic nucleotide phosphodiesterase activity should be expected to potentiate the stimulation of DNA synthesis and the cellular accumulation of cyclic AMP produced by cholera toxin. We found that addition of 0.05 mM 1-methyl-3-isobutylxanthine (IBMX) or 0.4 mM aminophylline, inhibitors of phosphodiesterase activity, markedly potentiated the stimulation of DNA synthesis promoted by cholera toxin in Swiss 3T3 cells (Fig. 3). These inhibitors of phosphodiesterase activity were shown to potentiate the effect of cholera toxin on cAMP levels in 3T3 cells (15,18).
Our findings indicate that an increase in the intracellular levels of cAMP acts synergistically with other mitogenic agents to stimulate DNA synthesis in Swiss 3T3 cells. This conclusion has been further substantiated by recent experiments showing that other agents that activate the adenylate cyclase and increase the intracellular level of cAMP, including adenosine agonists (18), prostaglandin E₁ (14, and manuscript in preparation), and isoproterenol (unpublished results), are also mitogenic for Swiss 3T3 cells maintained in serum-free medium. Finally, we recently found that cAMP derivatives butyryl cAMP or 8Br-cAMP stimulate DNA synthesis when added with insulin, phorbol esters, vasopressin, epidermal growth factor, or fetal bovine serum (19). The mitogenic effect of these agents is specific, because 8Br5'-AMP, 5'-AMP, or butyrate fails to stimulate DNA synthesis and because their mitogenic effects were markedly potentiated by the
Fig. 2. Time-course of stimulation of DNA synthesis in Swiss 3T3 cells by cholera toxin in the presence of 10 μg/mg insulin. The experimental conditions were as described in the legend to Fig. 1 except that the cumulative [3H]thymidine incorporation was terminated at various times as indicated.
Fig. 4. Effect of various concentrations of 8BrCAMP, 8BrCUMP, and 9Br5'-AMP on DNA synthesis in the presence of insulin. Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with Dulbecco's modified Eagle's (DME) medium at 37°C and incubated in 2 ml of a 1:1 mixture of DME and Waymouth medium containing [3H]thymidine (1 μCi/ml; 1 μM) and the additions as indicated. Insulin was added at 10 μg/ml. After 40 h at 37°C, the incorporation of radioactivity was determined as previously described (10,17).

Fig. 3. Dose-response of cholera toxin effect on stimulation of DNA synthesis in the absence (control + ) or presence of 0.4 mM aminophylline or 50 μM 1-methyl-3-isobutylxanthine (IMBX). Quiescent Swiss 3T3 cells were incubated in medium containing insulin at 10 μg/ml [3H]thymidine (1 μCi/ml; 1 μM) and various concentrations of cholera toxin for 40 h.
Since increased cAMP levels do not stimulate Na⁺ entry into quiescent cells (20, and Table 1), it appears that cAMP delivers a mitogenic signal to Swiss 3T3 cells through a mechanism not involving a primary increase in Na⁺-dependent ion fluxes. We conclude that cAMP and ion fluxes constitute separate mitogenic signals which are elicited by different sets of extracellular agents. In Swiss 3T3 cells neither of these signals, by itself, is sufficient to elicit a proliferative response in serum-free medium. However, when these signals (cAMP and Na-dependent ion fluxes) are induced simultaneously in quiescent 3T3 cells by an appropriate combination of extracellular factors, they act synergistically to stimulate exit from G₀ and entry into DNA synthesis. Although many aspects of this hypothesis require experimental verification and other growth-promoting signals may remain as yet undiscovered, this dual control of the initiation of DNA synthesis in 3T3 cells by identifiable intracellular signals, ion fluxes, and cAMP provides a model for understanding the organization and strategy of the mechanisms whereby extracellular agents may regulate animal cell proliferation.

Table 1. Uptake of Na⁺ into Swiss 3T3 cells in the absence or presence of cholera toxin and IBMX or of fetal bovine serum

Quiescent cultures of Swiss 3T3 cells were preincubated with 100 ng/ml cholera toxin and 50 μM IBMX for 90 min. Then, the cultures were shifted to DME medium containing 2 mM ouabain to prevent Na⁺ exit via the Na/K pump. Other parallel cultures were transferred to Dulbecco's modified Eagle's (DME) + 2 mM ouabain (control) or to DME + 2 mM ouabain containing 10% fetal bovine serum. After 30 min in the presence of ouabain the intracellular Na⁺ of all of the cultures was measured by flame photometry as previously described (5,11). The Na⁺ content of quiescent 3T3 cultures that had not been exposed to ouabain was 0.16 ± 0.04 μmol/mg protein and it was subtracted from the values obtained in the presence of ouabain to obtain the net Na⁺ uptake under the various conditions, as indicated. The figures in the table represent means ± S.D. and those in parentheses indicate the number of independent cultures used.

<table>
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<tr>
<th>Additions</th>
<th>Na⁺ uptake (μmol<em>mg protein⁻¹</em>30 min⁻¹)</th>
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<tr>
<td>None</td>
<td>0.15 ± 0.03 (n = 12)</td>
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<tr>
<td>Cholera toxin + IBMX</td>
<td>0.17 ± 0.03 (n = 12)</td>
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<tr>
<td>Fetal bovine serum</td>
<td>0.34 ± 0.01 (n = 8)</td>
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References