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Cell Biology

Vasopressin stimulation of mouse 3T3 cell growth

(DNA synthesis/growth control/epidermal growth factor/insulin/oxytocin)

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ABSTRACT **Vasopressin is shown to be a potent mitogen for Swiss 3T3 cells. The hormone (1–10 ng/ml) causes a striking shift of the dose–response curve for the effect of serum on thymidine incorporation by cultures of 3T3 cells arrested in the G_1/G_0 phase of the cell cycle. In the absence of added serum, the effect of vasopressin on DNA synthesis is greatly potentiated by insulin, epidermal growth factor, and a factor isolated from medium conditioned by simian virus 40-infected baby hamster kidney cells. The mitogenic effect of vasopressin is dependent on time and hormone concentration. In the presence of insulin, the half-maximal effect elicited by the peptide is obtained at 0.6 ng/ml. [Arg]Vasopressin and [Lys]vasopressin are equally potent. The vasopressins are 10^3-fold more potent than oxytocin. In the presence of a low (2.5%) concentration of serum, vasopressins stimulate cell proliferation.**

Many mammalian cells become arrested in the G_1/G_0 phase of the cell cycle when the culture medium is depleted of growth factors (1, 2). Addition of serum to such quiescent cultures stimulates a complex array of early biochemical events (3) and enhances DNA synthesis and cell division (1–3). One of the earliest changes produced by addition of serum (4, 5) or purified growth factors (6, 7) is an increase in the activity of the Na,K pump. Recently, it was found (8, 9) that the effect of growth-promoting factors on the pump is mediated by an increased entry of Na^+ into cells. The availability of Na^+ appears of critical importance in the regulation of the pump and possibly in the control of other cellular activities. These findings prompted us to test whether substances that are known to promote Na^+ entry can be mitogenic for quiescent cells. Pursuing this possibility, we found that vasopressins are potent mitogens for Swiss 3T3 cells.

MATERIALS AND METHODS

Cell Culture. Swiss mouse 3T3 cells (1, 10), propagated as described (4), were subcultured into 33-mm Nunc dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and were grown to confluence in this medium. All assays of growth-promoting activity were performed on such cultures. The cultures of 3T3 cells used in this study were arrested in the G_1/G_0 phase of the cell cycle. After exposure to [^{3}H]thymidine for 40 hr, less than 1% of the nuclei in the culture became radioactively labeled. Cell number was determined by removing the cells from the dish with a trypsin solution (0.05% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered salt solution with EDTA) and counting a portion of the resulting cell suspension in a Coulter Counter. Each point represents an average of at least two determinations.

Assays of Growth-Promoting Activity. All determinations of DNA synthesis were performed in a 1:1 mixture of Dulbecco's medium and Waymouth medium (11). The cultures were washed twice with Dulbecco's medium to remove residual serum immediately prior to assaying. For determinations of DNA synthesis, the medium (2 ml) contained either 0.2 μM [5 μCi (1 Ci = 3.7×10^{10} becquerels)/ml] or 2.5 μM (0.5 μCi/ml) [^{3}H]thymidine for autoradiography or incorporation into acid-precipitable DNA, respectively (4).

Materials. [Arg]Vasopressin and [Lys]vasopressin were obtained from Sigma. Mouse epidermal growth factor (EGF) was generously supplied by S. Cohen. Fibroblast-derived growth factor (FDGF) was isolated and purified from the medium conditioned by simian virus 40-infected baby hamster kidney cells as described (6). Bovine insulin (26 international units/mg) was obtained from Sigma. [^{3}H]Thymidine was from the Radiochemical Centre, Amersham, England. The serum used was fetal bovine (Flow Laboratories, Rockville, MD). All other materials used were of reagent grade.

RESULTS

Vasopressin added at 1 or 10 ng/ml caused a striking shift of the dose–response curve for the effect of serum on thymidine incorporation by cultures of 3T3 cells arrested in the G_1/G_0 phase of the cell cycle (Fig. 1). A marked synergistic effect was observed between the hormone and low (<5%) concentrations of serum that alone are incapable of producing a substantial stimulation of DNA synthesis. The requirement for serum could not be replaced by comparable concentrations of bovine serum albumin or ovalbumin.

Vasopressin interacted synergistically with polypeptide hormones such as EGF and insulin and with the purified FDGF. The synergistic interaction between vasopressin and the growth-stimulating factors occurred in serum-free, synthetic medium. Insulin potentiated the growth-promoting effect of vasopressin even at low concentrations (20–60 ng/ml), and the potentiation was more prominent at higher concentrations (Fig. 2). In addition, combinations of vasopressin with both insulin and EGF or insulin and FDGF stimulated DNA synthesis to a degree comparable to that achieved with medium containing 10% fetal bovine serum (Table 1). Dexamethasone (0.1–1 μg/ml) did not alter the response of 3T3 cells to vasopressin or to vasopressin and insulin (results not shown).

The striking synergistic interaction between vasopressin and insulin also could be demonstrated when the effects of the hormones were assessed as a function of time (Fig. 3). The combination of vasopressin and insulin stimulated DNA synthesis after a lag period of 12 hr which is identical to that produced by serum in the same experiment. The synergistic interaction results from a large change in the rate of entry into the S phase of the cell cycle. In separate experiments we found that removal of the vasopressin after 3 to 6 hr of incubation greatly decreased its mitogenic effect, suggesting that the hormone must be in contact with the cells for a considerable time before they become committed for DNA synthesis (Fig. 3 *inset*).

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Abbreviations: EGF, epidermal growth factor; FDGF, fibroblast-derived growth factor.

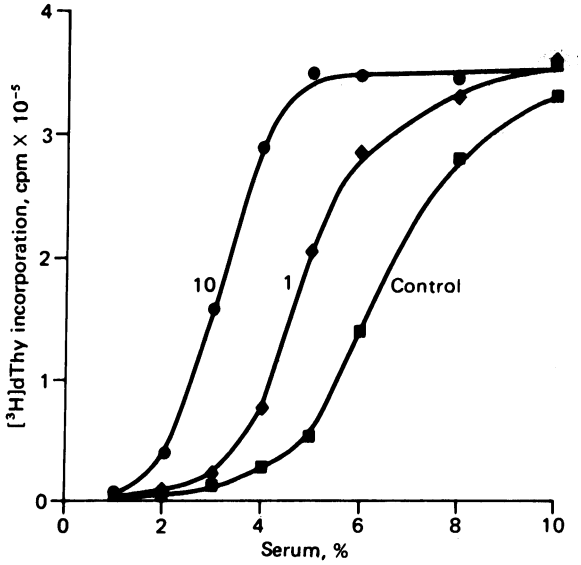

FIG. 1. Dose–response curves for the effect of serum on DNA synthesis by quiescent cultures of Swiss 3T3 cells in the absence or in the presence of vasopressin at 1 or 10 ng/ml. The serum and the hormone were added to the cultures in 2 ml of medium containing [^{3}H]thymidine. Incorporation of radioactivity into acid-insoluble material was determined 40 hr later.

The dependence of DNA synthesis stimulation on the concentration of vasopressin in the nutrient medium in the absence or presence of insulin is depicted in Fig. 4. Vasopressin elicited a maximal effect at a concentration of approximately 2 ng/ml; half-maximal effect was seen at about 0.6 ng/ml. The results were similar whether DNA synthesis was assessed by autoradiography of labeled nuclei or by incorporation of [^{3}H]thymidine into acid-insoluble material. In addition, a similar dependence of DNA synthesis on vasopressin concentration was obtained when FDGF was added instead of insulin (results not shown). Thus, the mitogenic potency of vasopressin in the presence of insulin is comparable to that of other growth factors such as EGF or FDGF (12).

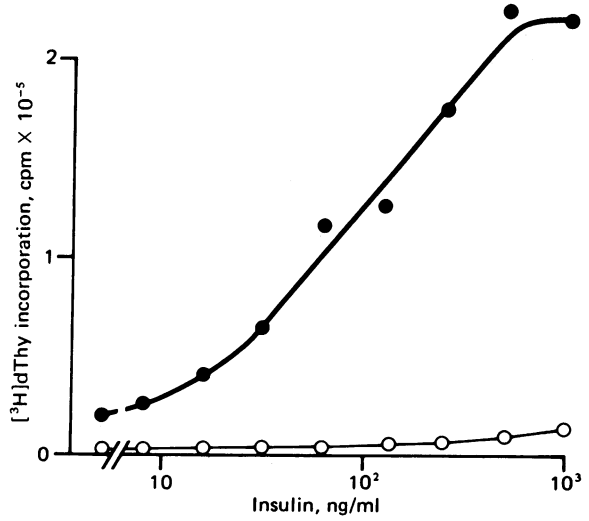

FIG. 2. Effect of various concentrations of insulin in the absence (○) or in presence (●) of vasopressin at 10 ng/ml on [^{3}H]thymidine incorporation by 3T3 cells. The cultures were exposed to hormones and radioactive substrate for 40 hr. In this experiment, 10% fetal bovine serum produced an incorporation into acid-insoluble material of 5×10^5 cpm per culture.

Table 1. Stimulation of DNA synthesis by combinations of hormones

Additions*	Vasopressin	% labeled nuclei
Insulin + EGF	−	17
Insulin + EGF	+	55
Insulin + FDGF	−	13
Insulin + FDGF	+	71
10% serum	−	74

* The concentrations of the hormones were 10 ng/ml, 1 μg/ml, 5 ng/ml, and 0.25 μg/ml for vasopressin, insulin, EGF, and FDGF, respectively. In the absence of factors or in the presence of individual hormones, the resultant labeled nuclei were <1%.

To determine whether 3T3 cells exhibit any specificity in their response to neurohypophyseal hormones, DNA synthesis stimulation as a function of oxytocin concentration is also shown in Fig. 4. Oxytocin was mitogenic for 3T3 cells but the concentration needed to produce half-maximal effect was ap-

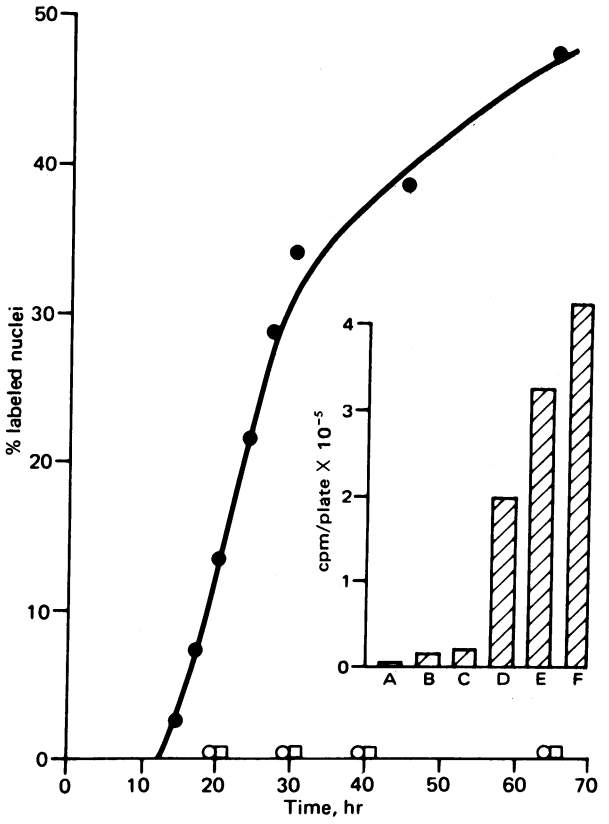

FIG. 3. Stimulation of DNA synthesis by vasopressin, insulin, or both as a function of time. The hormones were added at 0 hr. At the times indicated, dishes from each group were fixed and processed for autoradiography. The cultures were exposed to vasopressin at 10 ng/ml (○), to insulin at 1 μg/ml (□), or to both (●). Addition of medium containing 10% fetal bovine serum produced 76% labeled nuclei at 40 hr. (*Inset*) Effect of exposure to vasopressin for different times. Cultures were exposed to vasopressin (10 ng/ml) and insulin (1 μg/ml) in Dulbecco/Waymouth medium containing [^{3}H]thymidine for 3, 6, and 24 hr (bars B–D). At these times the cultures were washed five times with Dulbecco's at 37°C and the incubation was continued with identical medium except that vasopressin was omitted. Parallel cultures were exposed continuously to both hormones (bar E) or to medium containing 10% fetal bovine serum (bar F) or were incubated without stimulating agents (bar A) for 40 hr.

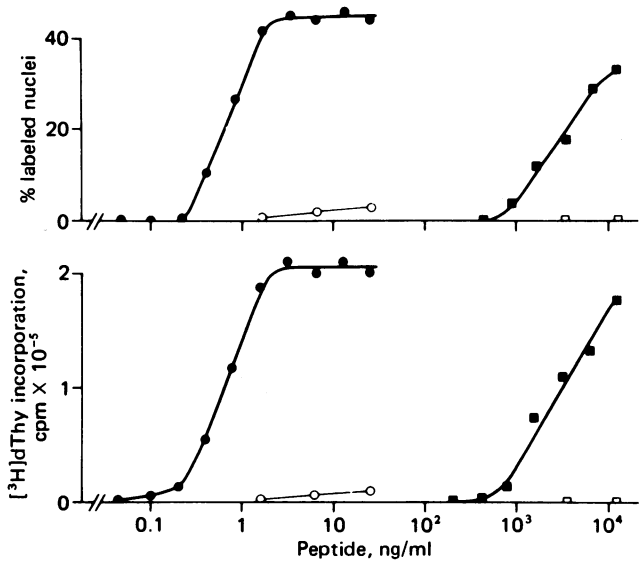

FIG. 4. Dose–response curves for the effect of vasopressin (●, ○) and oxytocin (■, □) on DNA synthesis by quiescent cultures of Swiss 3T3 cells in the absence (open symbols) or in the presence (solid symbols) of insulin. The hormones were added to confluent and quiescent cultures in 2 ml of Dulbecco/Waymouth medium containing [³H]thymidine. DNA synthesis was assessed either by autoradiography (*Upper*) or by incorporation into acid-soluble material (*Lower*) at 40 hr after continuous exposure to radioactive thymidine. In this experiment, 10% fetal bovine serum produced 84% labeled nuclei and 3.1 × 10^5 cpm per culture incorporated into acid-insoluble material.

proximately 2 μg/ml. In separate experiments, we found that the mitogenic potency of [Arg]vasopressin was comparable to that of [Lys]vasopressin. Clearly, 3T3 cells display a striking discrimination between the peptides; vasopressins are about 10^3-fold more potent than oxytocin in stimulating DNA synthesis in quiescent cultures of 3T3 cells.

The growth-promoting activity of vasopressin in cultures of 3T3 cells also could be demonstrated when cell proliferation was monitored over a period of several days. In the presence of 2.5% serum in the medium, a concentration of serum that does not support proliferation of our 3T3 cells, vasopressin at 10 ng/ml stimulated a 3-fold increase in cell number (Fig. 5*A*). When vasopressin was added with insulin (1 μg/ml), there was a clear synergistic effect resulting in a 6-fold increase in cell number. Vasopressin stimulated cell proliferation in a concentration-dependent manner; the effect was saturated at 6 ng/ml and a detectable stimulation was obtained at 0.8 ng/ml (Fig. 5*B*). The range of concentration that stimulated increases in cell number was somewhat higher than that required to stimulate DNA synthesis. The stimulation of cell proliferation by vasopressin could be demonstrated at different concentrations of serum (2–10%) in the medium (results not shown).

DISCUSSION

The present studies demonstrate that vasopressin is a potent mitogen for Swiss 3T3 cells. The hormone stimulates DNA synthesis and cell proliferation in the 1 ng/ml range, interacting synergistically with low concentrations of serum and with pure mitogens such as insulin, EGF, and FDGF in the absence of added serum. Both [Lys]vasopressin and [Arg]vasopressin are equally active, indicating that neither the lysine nor the arginine moiety is essential for the mitogenic effect of vasopressin in Swiss 3T3 cells. In contrast, the vasopressins are 10^3-fold more potent than oxytocin. Thus, the mitogenic effect of vasopressins in 3T3 cells displays a remarkable specificity.

In addition to Swiss 3T3 cells, secondary cultures of mouse embryo fibroblasts are also stimulated into DNA synthesis by vasopressin but the response is less marked (unpublished results). There is evidence indicating that vasopressin can be a mitogen for other cell types such as chondrocytes in cell culture (13) and bone marrow cells *in vivo* (14). It is also known that the small cell carcinoma of the lung produces large amounts of vaso-

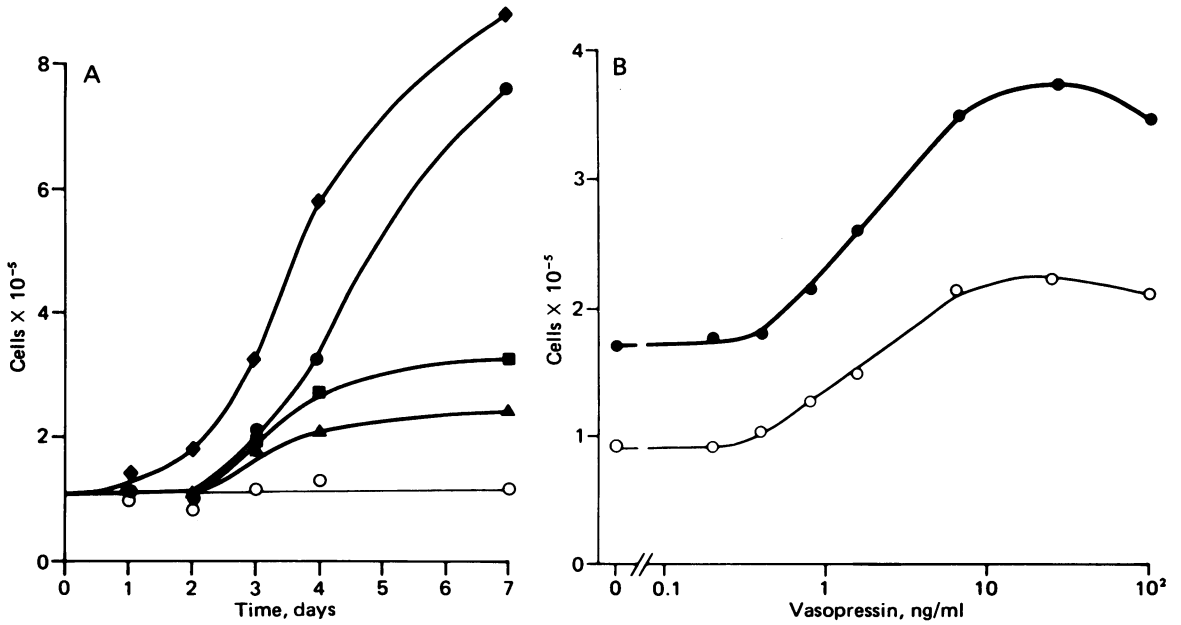

FIG. 5. (*A*) Effect of vasopressin, insulin, or both on the proliferation of 3T3 cells. The cells were seeded at 10^5 cells per 5-cm plastic dish containing 5 ml of Dulbecco/Waymouth medium supplemented with 2.5% fetal bovine serum (○). Some cultures received insulin (▲) at 1 μg/ml, vasopressin (■) at 10 ng/ml or both hormones (●). Growth of cultures in medium with 10% serum is also shown for comparison (♦). Cells were counted by removing them from the dish with a trypsin solution and counting a portion of the resulting suspension in a Coulter Counter. Each point represents an average of two determinations. (*B*) Dose–response curves for the effect of vasopressin on growth of Swiss 3T3 cells. The cells were seeded at 10^5 cells per 5-cm plastic dish containing 5 ml of Dulbecco/Waymouth medium supplemented with 2.5% fetal bovine serum. The cultures received different concentrations of vasopressin in the absence (○) or in the presence (●) of insulin (1 μg/ml). Cells were counted at 5 days after the start of the experiment.

pressin *in vivo* and *in vitro* (15, 16). In the light of the present findings on the mitogenic properties of vasopressin, it seems reasonable to ask whether the production of this peptide by such cells is linked with their abnormal proliferative properties.

The mitogenic activity of vasopressin, a hormone that promotes Na transport in various tissues, is consistent with the notion that ionic fluxes play a role in modulating the mitogenic response as recently suggested (8, 9). Furthermore, we have found that vasopressin rapidly accelerates influx of $^{22}Na^+$ and $^{86}Rb^+$ (a tracer of K^+) into quiescent cultures of 3T3 cells (unpublished results). However, other important metabolic effects of vasopressin such as changes in Ca^{2+} fluxes (17) or cyclic AMP concentration (18) might be involved in the mitogenic action of the hormone.

Vasopressin can provide a useful model peptide for further studies on the control of cell proliferation. An impressive number of structural analogs of vasopressin have been described and used to characterize the receptors (19, 20), and their use will help in defining the stereospecific requirements for stimulation of DNA synthesis and cell division. Furthermore, vasopressin can be isotopically labeled and still retain biological activity (19). A potent antiserum has also been produced (21). The vasopressin–3T3 cell interaction may provide a powerful tool for answering important questions concerning the mechanism by which peptide factors control cell proliferation.

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