Neurohypophysial-Hormone-Responsive Cell Line Derived From a Dimethylbenzanthracene-Induced Rat Mammary Tumour

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WRK-1, a cloned cell line derived from a rat mammary tumour, responds to physiological concentrations of vasopressin and pharmacological concentrations of oxytocin with increased incorporation of [^{14}C]acetate into lipids and increased protein accumulation. The presence of pharmacological concentrations of insulin, which itself is active on the WRK-1 cells, further enhances the effects of the neurohypophysial hormones. Unlike the action of vasopressin on other responsive tissues, the stimulation of acetate incorporation by WRK-1 cells is not observed until 24 h after the addition of the hormone. The lipids synthesized in response to the hormones are predominantly polar lipids, rather than the triacylglycerols characteristic of the differentiated mammary gland. [1-Deaminocysteine, 8-D-arginine]vasopressin, a vasopressin analogue that lacks pressor activity, has no effect on WRK-1 cells.

A variety of hormones have been shown to interact with the mammary gland in regulating growth and differentiation. These include oestrogens, androgens, progestins, glucocorticoids, prolactin, insulin, somatotropin (growth hormone), thyroxine and oxytocin. Many of these hormones have also been shown to interact with mammary cancers. We have reported the establishment of a cell line from a dimethylbenz[*a*]anthracene-induced rat mammary tumour whose apparent response to prolactin was due to contaminating vasopressin in the prolactin preparation (Kidwell *et al.*, 1978; Monaco *et al.*, 1978). We now describe the effects of vasopressin and oxytocin on this cell line and discuss implications of these findings.

Materials and Methods

Sodium [^{14}C]acetate (55–59 Ci/mol) was purchased from Amersham/Searle. [Arg⁸]vasopressin (385 i.u./mg), synthetic [Lys⁸]vasopressin and synthetic oxytocin were obtained from Calbiochem. Angiotensin II was purchased from Boehringer Mannheim. Insulin (mixture of porcine and bovine) was obtained from Eli Lilly Co. [1-Deaminocysteine, 8-D-arginine]vasopressin was obtained from Ferring Pharmaceuticals, Malmö, Sweden. Rat serum and foetal calf serum were obtained from Grand Island Biological Co.

Cell cultures

WRK-1 is a cloned cell line in long-term tissue culture originally derived from a dimethylbenzanthracene-induced rat mammary tumour. Morphological and growth characteristics of these cells have been previously described (Kidwell *et al.*, 1978). In brief, they grow in monolayer culture and appear to possess many of the ultrastructural characteristics of mammary secretory epithelium. Their epithelial nature is further supported by their ability to grow in D-valine. They do not have receptors for oestrogens, androgens, progesterone or prolactin. They do possess glucocorticoid receptors (Lippman, M. E. & Monaco, M. E., unpublished work). Although they do not form tumours when injected into nude mice, they appear to be transformed. They have a modal chromosome number of 80, and 'pile up' in culture. The cells were cloned in the following manner. They were dispersed and diluted to give a final concentration of one cell/ml, and 1 ml samples were then transferred to small plastic wells. Wells that were observed under the microscope to contain only a single cell were marked, and the resulting outgrowths constituted cloned lines. For hormone-stimulation experiments, confluent dishes of cells were harvested in trypsin and replicately plated into multiwell plastic dishes each containing 2 ml of Eagle's minimal essential medium (N.I.H. Media Unit) in 10% (v/v) foetal calf serum and 2% (v/v) rat serum. When the cell density reached about half

confluency (3×10^5 – 4×10^5 cells), the medium was changed to one without serum. After 6 h, the medium was replaced with 2 ml of fresh serum-free medium and hormones were added.

Measurement of acetate incorporation

At designated times after hormone addition, cells were labelled for 1 h with $20 \mu\text{M}$ - $[^{14}\text{C}]$ acetate ($1 \mu\text{Ci}/\text{ml}$) at 37°C in an incubator gassed with air/ CO_2 (19:1). At the end of the labelling period, the medium was removed and the cells were harvested with 1 ml of 0.02% (w/v) EDTA in Dulbecco's phosphate-buffered saline (N.I.H. Media Unit) without calcium or magnesium, pH 7.4. Cell pellets were collected by centrifugation at high speed in a Clay-Adams Serofuge. The resulting pellets were resuspended in phosphate-buffered saline and samples taken for protein determinations (Lowry *et al.*, 1951) using bovine serum albumin as standard. The remaining cells were recentrifuged as above and the pellet was incubated in 0.1 ml of 30% (w/v) KOH for 2 h at 90°C in order to saponify the triacylglycerols and phospholipids. The samples were then cooled, acidified with 0.1 ml of HCl, and the fatty acids extracted with 8 vol. of hexane. The fatty acid nature of the labelled compounds was verified by t.l.c. on silica gel G in hexane/diethyl ether/acetic acid (280:120:1, by vol.). The chain lengths of the fatty acids synthesized have been previously described in detail (Kidwell *et al.*, 1978). A portion of the hexane extract was transferred to a glass scintillation vial to which 8 ml of Aquasol (New England Nuclear) was added. A Packard Tri-Carb (model 3390) was used to quantify the radioactivity.

Identification of labelled lipids

The total lipid fraction was extracted from the cells with 8 vol. of chloroform/methanol (2:1, v/v). The classes of lipids were identified by t.l.c. of this extract on silica gel G in hexane/diethyl ether/acetic acid (1400:600:1, by vol.). Details of the procedure have been previously described (Monaco & Lippman, 1977).

Results

Effect of vasopressin and insulin on acetate incorporation by WRK-1 cells

Fig. 1 illustrates the effects of maximally stimulatory concentrations of vasopressin and insulin on acetate incorporation into lipids. Both vasopressin and insulin alone stimulate incorporation by 2-fold and 10-fold respectively in this experiment. The two effects are synergistic, increasing acetate incorporation to 30-fold that in controls. In general, overall inductions range between 10- and 50-fold for vasopressin plus insulin and appear to vary as a

Fig. 1. Effects of vasopressin and insulin on the incorporation of $[^{14}\text{C}]$ acetate into lipids by WRK-1 cells. Methods were as described in the text. Cells were labelled after 36 h of treatment with serum-free medium alone, serum-free medium plus vasopressin (1 nM) and/or serum-free medium plus insulin (1 μM). Results given are means $\pm$ 1 S.D. of triplicate determinations.

function of the density of the cells, with greater inductions apparent at lower densities (results not shown).

Vasopressin-dose-response curves

The dose-response curve for vasopressin in the presence of a maximal concentration of insulin is shown in Fig. 2. Stimulation is observed with as little as 80 pM- $[\text{Lys}^8]$ vasopressin ($20 \mu\text{i.u.}/\text{ml}$). Physiological concentrations of vasopressin range from 4 to $45 \mu\text{i.u.}/\text{ml}$ (Lauson, 1967); therefore the lower dose ($20 \mu\text{i.u.}/\text{ml}$) is well within the physiological range. Results were qualitatively identical in the absence of insulin (not shown). Treatment with vasopressin alone resulted in a mean induction of 3.70-fold (S.E.M. $\pm$ 0.09) for 20 separate experiments.

Time course of vasopressin stimulation of acetate incorporation

Fig. 3 shows the time course of stimulation by vasopressin in the presence of insulin of acetate incorporation by WRK-1 cells. There is a definite lag; no increase in incorporation is seen at 10 h and maximal stimulation is not observed until 36 h after the addition of the hormone. As in the dose-response curve, the absence of insulin had no effect on the time course (results not shown). Furthermore, similar results were obtained when the cells were preincubated with insulin for 24 h to allow for a maximal effect of insulin before the addition of vasopressin.

Fig. 2. Effect of vasopressin on the incorporation of [¹⁴C]acetate into lipids by WRK-1 cells
Methods were as described in the text. Each dish contained 1 μM-insulin. Cells were labelled after 36 h of hormone treatment. Results given are the means ± 1 S.D. of triplicate determinations.

Fig. 3. Time course of vasopressin stimulation of the incorporation of [¹⁴C]acetate into lipids by WRK-1 cells
Vasopressin concentration was 1 nM. Methods were as described in the text. Each dish contained 1 μM-insulin. Results given are the means of triplicate determinations. Standard deviations ranged from 16 to 23%.

Identification of labelled lipids

Table 1 outlines the proportions of various labelled-lipid classes (polar, neutral and non-esterified fatty acids) present in the chloroform/methanol extract. In all cases the labelled lipids are predominantly polar lipids; however, the presence of hormones, especially vasopressin, appears to increase the relative proportion of these polar lipids.

Effect of hormones on protein accumulation

Because the radiolabelled lipids synthesized by these cells in response to vasopressin are pre-

Table 1. Classes of lipids synthesized by WRK-1 cells
Methods are described in the text. Values in parentheses are the results of a second identical experiment.

Addition	Composition (%)		
	Polar lipid	Non-esterified fatty acids	Neutral lipid
None	43 (53)	31 (26)	26 (20)
Vasopressin	75 (80)	21 (16)	4 (2)
Insulin	66 (65)	20 (28)	14 (5)
Vasopressin + insulin	63 (76)	20 (18)	5 (4)

Fig. 4. Effects of vasopressin and insulin on the accumulation of protein by WRK-1 cells
Methods were as described in the text. Hormone concentrations are as follows: vasopressin, 1 nM; insulin 1 μM. Results given are the means of triplicate determinations. The mean standard deviation was 11%.

dominantly those used by cells in the synthesis of membranes (polar lipids), we wondered whether or not the hormones also have other effects on the overall growth of the cells. Fig. 4 compares the total protein per dish in control cells and cells treated with vasopressin and/or insulin. Both insulin and vasopressin alone increase protein accumulation by about 100% at 72 h, and the two hormones together act additively to produce a 200% increase above control values.

Specificity of the response to vasopressin

Fig. 5 compares dose-response curves for a variety of peptide hormones with activities similar to

Fig. 5. Comparative potencies of antidiuretic, pressor and oxytocic peptides in stimulating acetate incorporation into lipids by WRK-1 cells

Methods were as described in the text. Each dish contained $1 \mu\text{M}$ -insulin. ●, [Lys]vasopressin; □, [Arg]vasopressin; ○, oxytocin; △, angiotensin II; ▲ [1-deaminocysteine, 8-D-arginine] vasopressin. Results given are the means of triplicate determinations. The mean standard deviation was 13%.

vasopressin. [Arg⁸]vasopressin, the naturally occurring vasopressin in rats, is at least as active as [Lys⁸]vasopressin. Oxytocin, which normally interacts with the myo-epithelium of the mammary gland, also interacts with WRK-1 cells; however, 100–1000-fold higher concentrations are required to elicit a response. The effects of vasopressin and oxytocin are not additive. [1-Deaminocysteine, 8-D-arginine]vasopressin, a synthetic peptide that possesses an antidiuretic/pressor ratio of 4000:1 (Robinson, 1976), is totally inactive; however, angiotensin II, a potent pressor agent, is also inactive. The presence of [1-deaminocysteine, 8-D-arginine]vasopressin does not inhibit the response to [Lys⁸]vasopressin and is therefore neither an agonist nor antagonist in this system.

Discussion

WRK-1 is a cloned cell line derived from a dimethylbenzanthracene-induced tumour which responds to physiological concentrations of vasopressin and pharmacological concentrations of oxytocin and, as such, provides a model system for studying the mechanism of action of these hormones *in vitro*. A concentration of vasopressin of 80 pM (20 $\mu\text{i.u./ml}$) elicits a response in these cells. Insulin also stimulates WRK-1 cells, and when vasopressin and insulin are added together, a synergistic response is observed. However, a concentration of insulin of higher than 0.1 nM is required to elicit a response

(results not shown), suggesting that this is not a physiologically relevant action of the hormone; that is, insulin may interact with a receptor for another related peptide hormone.

Unlike other actions of vasopressin on toad bladder (Zusman *et al.*, 1977; Weismann *et al.*, 1976), kidney (Roy *et al.*, 1977) and liver (Hems & Whitton, 1973; Kirk *et al.*, 1977), the lag time before the effect of vasopressin on acetate incorporation into lipids can be seen is relatively long. A minimal effect is observed at 24 h, and maximal inductions are not apparent until 36 h of hormone treatment. A similar delay is seen in the effect of the hormone on protein accumulation. However, it is possible that some other effect of the hormone on these cells is more rapid.

The stimulation of acetate incorporation by vasopressin and insulin results in increased proportions of radiolabelled polar lipids, although the nature of the individual fatty acids remains unchanged (Kidwell *et al.*, 1978). A similar effect of vasopressin on the incorporation of radiolabelled phosphate into phospholipids has been reported in liver (Kirk *et al.*, 1977). We have previously reported that treatment of these cells with hormones results in an increase in neutral-lipid droplets present within the cells (Kidwell *et al.*, 1978), and these data support that finding. Although the proportion of neutral lipids decreases from 29 to 5% with hormonal stimulation, there is nevertheless an increase in the absolute amount of neutral lipid present.

Polar lipids are utilized by cells in the synthesis of membranes, suggesting that vasopressin may act as a growth stimulator in these cells. The increase in total protein accumulation observed in the presence of the hormone supports this idea.

In addition to providing a model system for the study of neurohypophysial-hormone action, the response of these cells to vasopressin and oxytocin raises several questions. The first concerns the nature of the WRK-1 cells. Whereas in many respects they closely resemble secretory mammary epithelium (Kidwell *et al.*, 1977) and lack myo-epithelial characteristics, such as myofibrils, their response to neurohypophysial hormones suggests that they may nevertheless be of myoepithelial origin. Interactions of vasopressin with contractile tissue have been extensively documented. (1) Vasopressin has one-fifth the potency of oxytocin in the assay *in vivo* of rat mammary-gland milk ejection (van Dyke *et al.*, 1955); in the assay of milk ejection *in vitro*, vasopressin and oxytocin are equipotent (van Dongen & Marshall, 1967). (2) Vasopressin stimulates contraction of the uterus, and in some cases, such as the human non-pregnant uterus (Coutinho & Lopes, 1968), is a more potent agonist than oxytocin. (3) Vasopressin initiates contraction

of arterial smooth muscle (Oliver & Shafer, 1895). The inability of [1-deaminocysteine, 8-D-arginine]-vasopressin to stimulate mammary-gland myoepithelial contraction has been documented (3.2 i.u./mg) (Vavra *et al.*, 1968). Thus the failure of [1-deaminocysteine, 8-D-arginine]vasopressin to stimulate WRK-1 cells is consistent with the notion that the effects of vasopressin on these cells reflects a myotonic rather than an antidiuretic action of the hormone. Although it is possible that WRK-1 cells are derived from vascular smooth-muscle rather than mammary-gland myoepithelium, the nature of the dose-response curve (WRK-1 cells respond to low concentrations of vasopressin) and the inability of angiotensin II, a potent pressor agent that has no activity in the milk-ejection assay (Brovetto *et al.*, 1967), to stimulate the cells supports the myoepithelial hypothesis.

The relative potencies of oxytocin and vasopressin on acetate incorporation are the reverse of those seen in milk-ejection assays; that is, oxytocin is the more potent agonist physiologically during lactation. However, relative potencies have never been measured (and cannot be measured) under non-lactating conditions; therefore it is possible that the hormonal environment of pregnancy and/or lactation is necessary to induce full responsiveness to oxytocin. Biological assays indicate that this is indeed the case for the human uterus (Coutinko & Lopes, 1968), and binding studies with labelled oxytocin in rabbit uterus and rat mammary gland suggest that the increased sensitivity during pregnancy and/or lactation is a result of an increase in receptor number (Nissenson *et al.*, 1978; Soloff *et al.*, 1979).

However, there is also a third possibility, namely that the response of WRK-1 cells to vasopressin is a result of the malignant nature of the cells. Thus the second question raised by these data concerns the role of neurohypophysial hormones, if any, in the induction and growth of mammary cancers. Mitogenic effects of vasopressin on HeLa cells (Bernard-Weil & Da Lage, 1968) and on 3T3 cells (Rozenfurt *et al.*, 1979) have been reported. Furthermore, carcinogen-induced rodent mammary tumours are often heterogeneous, and proliferating myoepithelial components have been described (Tarin, 1969). Some human mammary tumours have also been described as proliferative myoepithelium, although there is controversy concerning these assignments (Murad & Scarpelli, 1967; Kermarec *et al.*, 1973; Fisher, 1976). Lastly, a clinical trial designed to inhibit endogenous vasopressin followed by glucocorticoid administration has been successful in the palliation of metastatic breast cancer (Bernard-Weil & Pilleron, 1973). However, the role of purported vasopressin inhibition in the subsequent remissions is unclear.

In summary, WRK-1, a cell line derived from a dimethylbenzanthracene-induced mammary tumour and possibly of myoepithelial origin, responds to neurohypophysial hormones, and, as such, provides a convenient model system for the study of the mechanism of action of these hormones. In addition, the responsiveness of WRK-1 cells provokes questions about the role of neurohypophysial hormones in the growth of mammary cancer *in vivo*.

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