Although previous experiments have not shown exaggerated steroid feedback effects after MSG-treatment[7,14] the present results agree with the corroborative data on hypothalamic LHRH stores and LHRH receptors. It is suggested that while the destruction of the tuberoinfundibular dopaminergic system[2], or other calecholaminergic or peptidergic neurones[15], after MSG treatment reduces basal release of LHRH, the hypothalamus can be triggered to release substantial amounts of LHRH provided it is challenged with appropriate steroid feedback signals. The resultant LH surge is consequently amplified by the accumulated stores of LHRH which acts on a pituitary gland that has retained normal levels of LHRH receptors and responsiveness to LHRH, despite the reduction in gonadotroph size.

Acknowledgments. We are grateful to Dr P. McGill (Hoechst (U.K.) Ltd.) for the gift of buserelin, to Dr H. Fraser for LHRH antiserum, to the National Hormone and Pituitary Agency for radioimmunoassay materials, and to the Wellcome Trust and Vandervell Foundation for financial support.

1 Inkster, S.E., Clayton, R.N., and Whitehead, S.A., J. Endocr. *107* (1985) 9.
2 Nemeroff, C.B., Konkol, R.J., Bissette, G., Youngblood, W., Martin, J.B., Brazeau, P., Rone, M.S., Prange, A.J., Breese, G.R., and Kizer, J.S., Endocrinology *101* (1977) 613.
3 Spinedi, E., Johnston, C., and Negro-Vilar, A., Endocrinology *115* (1984) 267.
4 Badger, T.M., Millard, W.J., Martin, J.B., Rosenblum, P.M., and Levenson, S.E., Endocrinology *111* (1982) 2031.
5 Greeley, G.H., Nicholson, G.F., and Kizer, J.S., Brain Res. *195* (1980) 111.
6 Sridaran, R., Rodriguez-Sierra, J.F., and Blake, C.A., Proc. Soc. exp. Biol. Med. *168* (1981) 38.
7 Clemens, J.A., Roush, M.E., Fuller, R.W., and Shaar, C.J., Endocrinology *103* (1978) 1304.
8 Clayton, R.N., Solano, A.R., Garcia-Vela, A., Dufau, M.L., and Catt, K.J., Endocrinology *107* (1980) 644.
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
10 Jeffcoate, S.L., Fraser, H.M., Holland, D.T., and Gunn, A., Acta endocr. (Copenh) *75* (1974) 625.
12 Dada, M.O., Rodriquez-Sierra, J.F., Clough, R.W., Garner, L.L., and Blake, C.A., Endocrinology *116* (1985) 246.
13 Dada, M.O., and Blake, C.A., J. Endocr. *104* (1985).
14 Dyer, R.G., Weick, R.F., Mansfield, S., and Corbet, H., J. Endocr. *91* (1981) 341.
15 Kreiger, D.T., Liotta, A.S., Nicholson, G., and Keizer, J.S., Nature *278* (1979) 562.

0014-4754/87/060606-03$1.50 + 0.20/0

17β-Estradiol-sensitivity of cultured myometrial cells

J.F. Krall

The Biochemical Pharmacology Laboratory (111K), and the UCLA-SFVP Department of Medicine, VA Medical Center, 16111 Plummer Street, Sepulveda (California 91343, USA), 10 September 1986

Summary. The estrogen sensitivity of cells cultured from the rat myometrium was studied by growing the cells in the absence or presence of 1 nM 17β-estradiol. Following a time lag of ~ 10 days, exposure to estrogen resulted in increased incorporation of radiothymidine by the cells. Estrogen treatment also decreased isoproterenol-dependent and GTP-dependent adenylate cyclase activity, but had no effect on basal activity. These cultured cells have been shown previously to have some properties of uterine smooth muscle. The effects estrogen has in vitro, therefore, may reflect important properties in vivo that account for the mechanism by which the sex steroid decreases the sensitivity of the myometrium to isoproterenol.
Key words. β-Adrenergic catecholamines; estrogen; cAMP synthesis; myometrium.

The sex steroid hormones have well characterized effects on the patterns of uterine motility and their regulation by adrenergic catecholamines[1]. Estrogen (E_2) in general, has been associated with both an increase in uterine contractions and a decrease in sensitivity to the relaxing effects of β-adrenergic catecholamines[2,3]. The characterization of the mechanism that explains the effect of E_2 on β-adrenergic catecholamine sensitivity has remained ellusive.

Although E_2 decreases myometrial sensitivity, it increases β-adrenergic receptors in the rat[4]. One reason for these disparities may be that E_2 effects more than one cell type in vivo[3,5]. This complexity might be circumvented in tissue culture using myometrial cells with more homogeneous properties, and smooth muscle cells from the myometrium have been cultured in several laboratories. Although the cultured cells may lose their contractile properties, they retain other important properties of uterine smooth muscle cells. These include sensitivity to oxytocin and relaxin and receptors for acetylcholine and β-adrenergic catecholamines[6-9].

Myometrial cells growing in tissue culture also retain E_2 receptors and nuclear estrogen-binding sites[10]. Sex steroid sensitivity in vitro might include effects on β-adrenergic receptor-mediated cAMP production which could reflect the way E_2 effects uterine motility in vivo.

Materials and methods. Cell culture. Smooth muscle cells were obtained from the uteri of 21-day-old Sprague-Dawley rats and grown in cell culture as described by Fortier et al.[7]. Cells were grown to the stage of confluent monolayers in growth medium with normal serum, then changed to the same medium with charcoal extracted medium. Cells passaged in culture 4–6 times were used in the experiments described here.

Radiothymidine incorporation. 17β-Estradiol (E_2) was added on successive days at a final concentration of 1 nM to cultures which had been maintained in medium containing charcoal extracted serum. The cell culture medium was changed every 4 days and during these routine changes fresh E_2 was added to the cultures which had been growing in it previously. At the culmination of the experiment, which lasted up to 35 days, the medium was discarded and all of the cultures received fresh medium which contained 10 μCi per ml [^{3}H]thymidine (NEN, Boston). Incorporation of the radioisotope was allowed to continue for 3 h after which the radioactive medium was discarded and the cultures washed 3 times with phosphate buffered saline.

The contents of each culture dish was suspended with the aid of a plastic scraper in 1 ml of 0.2 N NaOH and the suspensions heated at 90 °C for 15 min. The solubilized contents were chilled and an equal volume of ice cold 20% TCA was added. The acid insoluble fraction was allowed to precipitate overnight at 4 °C. The precipitates were collected by vacuum filtration onto glass fiber filters, washed with 5% ice cold TCA, dried, dissolved in 90% NCS (Amersham) and counted in toluene based scintillation fluid.

Adenylate cyclase activity. In parallel experiments, cells were incubated in the absence or presence of estradiaol for 8 days then

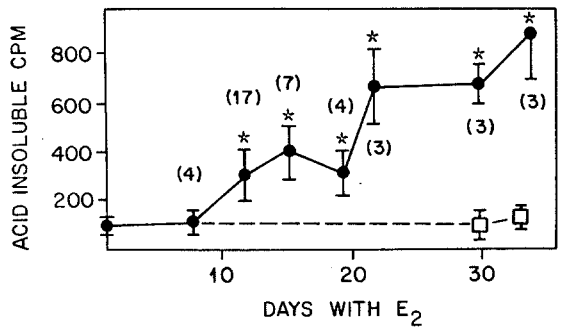

Figure 1. Effect of exposure to E_2 on smooth muscle cell [^{3}H]thymidine incorporation in vitro. The cultures were continuously exposed to 1 nM E_2 for the number of days indicated during the course of the 35-day culture period before the determination of [^{3}H]thymidine incorporation as described in 'Materials and methods'. The results are the mean±SEM, of the number of separate determinations at each time point shown in parenthesis. In parallel experiments, confluent cultures were changed to charcoal extracted serum 5 days and 1 day before they were incubated with [^{3}H]thymidine under the conditions described above (□). *, significantly ($p < 0.05$) greater than incorporation by cells cultured for the entire experiment in the absence of E_2 (0 days with E_2) when compared using an unpaired t-test.

washed as described above. Adenylate cyclase activity in these cells was determined by the method we described previously[7]. Briefly, the cells were harvested by scraping in adenylate cyclase homogenization buffer (0.05 M Na^+-HEPES, pH 7.6; 0.001 M EGTA, and 10% DMSO). The cell suspensions were homogenized by hand in a teflon-glass homogenizer and the homogenate stored frozen at −90 °C and assayed within two weeks for enzyme activity. Adenylate cyclase activity was determined as the enzymatic conversion of [α-^{32}P]ATP (NEN, Boston) to [^{32}P]cAMP followed by purification of the radioactive product by Dowex-aluminium oxide chromatography after the method of Salomon et al.[11].

The protein concentration of the homogenates was determined by the method of Lowry et al.[12] using BSA as standard.

Results. Confluent cultures were exposed to E_2 in medium which contained charcoal extracted serum. During these experiments, the cell culture medium was replenished every 4 days and fresh E_2 was added during these routine changes of medium. The

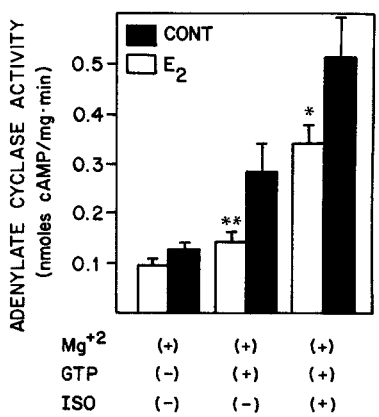

Figure 2. Effect of E_2 exposure on isoproterenol-dependent adenylate cyclase activity. Cells grown in absence (CONT) or presence of 1 nM E_2 for 14 days were harvested and adenylate cyclase activity determined as described in 'Materials and methods'. Additions to the enzyme assay were 10 mM $MgCl_2$, 300 µM GTP and 100 µM isoproterenol (ISO) in the combination indicated. The results are the mean±SEM of four different experiments. *, **, significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the control values when compared using an unpaired t-test.

levels of E_2 detectable in the medium by radioimmunoassay did not change during these 4-day intervals (data not shown). Exposure to E_2 resulted in an increase in [^{3}H]thymidine incorporation by the cultured cells (fig. 1). The increase followed an appreciable time lag and required up to 10 days of continuous exposure to E_2. After the initial time lag, [^{3}H]thymidine incorporation remained elevated during the subsequent course of exposure to the steroid.

These results indicated the cultured smooth muscle cells had retained E_2 sensitivity as they adapted to the cell culture conditions. Additional effects of exposure to E_2 were investigated at the level of the β-adrenergic receptor as reflected by isoproterenol-dependent adenylate cyclase activity. These determinations were carried out using cells continuously exposed to the medium containing charcoal extracted serum in either the presence or absence of 1 nM E_2 for 18 days. Despite the significant increase ($p < 0.05$) in [^{3}H]thymidine incorporation by the E_2-treated cells over this period (fig. 1), there was no significant ($p > 0.05$) change in cell number (data not shown).

Exposure to E_2 under the conditions described did result, however, in a significant ($p < 0.05$) decrease in adenylate cyclase activity determined in the presence of Mg^{2+}-GTP and in the presence of Mg^{2+}-GTP plus isoproterenol (fig. 2). As shown previously[7], isoproterenol stimulates adenylate cyclase activity in these cultured cells in the presence but not in the absence of GTP.

In contrast to the GTP-dependent components of adenylate cyclase activity that represent properties of the coupled β-adrenergic receptor, exposure to E_2 had no significant effect ($p > 0.05$) on basal activity, determined in the presence of Mg^{2+} alone (fig. 2). When it was added directly to the enzyme assay, estrogen had no effect on adenylate cyclase in either the absence or presence of GTP or isoproterenol (data not shown).

Discussion. Cells cultured from the rat myometrium were sensitive in vitro to the addition of low concentrations of E_2 as reflected by an increase in the rate of radiothymidine incorporation. This effect of E_2 exposure was slow and occurred following a week-long time lag. It was, therefore, probably related to E_2 effect on the initiation of cell growth rather than rapid effects of the steroid on cell permeability to the radioisotope. Cultured cells like these have been shown previously to have properties of myometrial smooth muscle. In the intact myometrium, E_2 treatment is associated with increased contractility and refractoriness to the relaxing effects of β-adrenergic catecholamines[3]. As expected from its effects on uterine motility, E_2 decreases isoproterenol-dependent cAMP production when it is administered to ovariectomized rats[13].

The mechanism of the loss of sensitivity is unknown, however. It occurs despite the fact that E_2 exposure leads to an increase in β-adrenergic receptors in the rat myometrium[4]. In the experiments described here, E_2 affected isoproterenol-dependent cAMP production in a manner that was qualitatively similar to effects the steroid has in vivo. Besides decreased isoproterenol-dependent cAMP synthesis, E_2 treatment also decreased GTP-dependent but not basal adenylate cyclase activity, a reflection of effects the steroid had at the level of the N_s regulatory protein. This change was not observed when E_2 was added directly to the enzyme assay and suggests, therefore, that E_2 treatment slowly alters cAMP production by cultured uterine smooth muscle cells. The effect E_2 had on adenylate cyclase activity was not associated with an increase in cell number over the period we studied. It may have been related, however, to the initiation of growth as reflected by increased [^{3}H]thymidine incorporation in charcoal extracted serum, conditions that failed to support real growth in the cultures.

While speculative in the absence of more definitive evidence, the decrease in isoproterenol responsiveness might be attributed to steroid-dependent effects at the level of the N_s regulatory protein since guanine nucleotide-dependent adenylate cyclase activity was preferentially affected. This is the level at which the β-adre-

nergic receptor is coupled with the adenylate cyclase catalytic unit[14]. Myometrial cells in vitro share some other properties with uterine smooth muscle cells in vivo. Results presented here suggest but do not prove that E_2-sensitivity of β-adrenergic receptor-mediated cAMP synthesis may be one of these.

Acknowledgments. This study was supported by funds from the Veterans Administration.

1 Krall, J., and Korenman, S., in: Advances in Sex Hormone Research, vol. 3, p. 75. Eds J. A. Thomas and R. L. Singhal. University Park Press, Baltimore 1977.
2 Marshall, J., Am. J. Physiol. *197* (1959) 935.
3 Miller, M., and Marshall, M., Am. J. Physiol. *209* (1965) 859.
4 Krall, J., Mori, H., Tuck, M., LeShon, S., and Korenman, S., Life Sci. *23* (1978) 1073.
5 Cruz, M., and Rudolph, M., Life Sci. *38* (1986) 2043.
6 Hsu, C., McCormack, S., and Sanborn, B., Endocrinology *116* (1985) 2029.
7 Fortier, M., Chase, D., Korenman, S., and Krall, J., Am. J. Physiol. *245* (1983) C84.
8 Hsu, C., and Sanborn, B., Endocrinology *118* (1986) 495.
9 Krall, J., and Morin, A., J. Cell Physiol. *129* (1986) 250.
10 Markaverich, B., Roberts, R., Alejandro, M., and Clark, J., J. biol. Chem. *261* (1986) 142.
11 Salomon, Y., Londos, C., and Rodbell, M., Analyt. Biochem. *58* (1974) 541.
12 Lowry, O., Rosebrough, N., Farr, A., and Randall, R., J. biol. Chem. *193* (1951) 265.
13 Chew, C., and Rinard, G., Biochim. biophys. Acta *362* (1974) 493.
14 Gilman, A., J. clin. Invest. *73* (1984) 1.

0014-4754/87/060608-03$1.50 + 0.20/0

Differentiation of L6 myoblastic cells into chondrocytes

M. Bettex-Galland and U. Wiesmann

Department of Paediatric Surgery, University of Berne, CH-3010 Berne (Switzerland), 16 September 1986

Summary. Under the influence of demineralized bone pieces L6 cells differentiate into chondrocytes. The cartilage formed is identifiable histologically. The results demonstrate that these myoblastic cells, which are committed to produce muscle, may still be influenced to express another potentiality of their genome.
Key words. Demineralized bone matrix; cell culture; L6 myoblastic cells; chondrocytes; chondrogenesis; cellular differentiation.

The implantation in animals of demineralized bone matrix (DBM), generally prepared in pulverized form, induces in a first step the formation of cartilage, even in ectopic sites, i.e. under the skin or intramuscularly. The test animal is usually the rat, and optimal results are mainly obtained with allogenic implants. After invasion of the implant by blood capillaries, bone marrow forms, and around it, osteoblasts begin to secrete osteoid, which subsequently mineralizes. A regular cancellous ossicle is formed[1].

It is generally assumed that the cells from which chondrocytes originate under the influence of a cytokine contained in DBM, are undifferentiated mesenchymal cells.

In vitro, upon incubation of embryonic rat muscle with rat DBM pieces, prepared by cutting demineralized bones longitudinally, chondrocytes secreting cartilage matrix appear[2,3]. As angiogenesis cannot take place under these conditions, no bone is formed.

Thompson, Piez and Seyedin[4] did not detect any proteoglycan synthesis in 14-day cultures of DBM extracts with L6 cells, assayed for cartilage proteoglycan by an ELISA, although mesenchymal cells prepared from rat embryonic muscle and incubated under the same conditions consistently gave a positive result. On the contrary, Glowacki and Mulliken[5] observed incorporation of ^{35}S-sulfate into chondroitin sulfate by cells of mesenchymal origin, i.e., 3T3, L6 and swine aorta smooth muscle cells. A discrepancy exists between the above mentioned results which could be due to differences in sensitivity between the methods used.

On the other hand, as the implantation of pulverized DBM into the rat abdominal rectus muscles consistently elicits the formation of cartilage and bone[6], the possibility of a differentiation of chondrocytes from myoblastic cells seems worth testing. Therefore, we have tried in a series of experiments to find out whether L6 cells, which behave characteristically as myoblasts, would express a chondrocytic phenotype as a result of incubation with rat DBM pieces.

Experimental procedures. L6 cells were purchased from ATCC (number CRL 1458). The culture medium was essentially the same as that utilized by Nathanson and Hay[3]: CMRL 1066, with 15% heat inactivated fetal calf serum, containing penicillin (100 units/ml) and streptomycin (100µg/ml). Incubation was at 37 °C in an atmosphere containing 5% CO_2.

Demineralized bone pieces were prepared from the humerus, femur and tibia of adult male Wistar rats killed with Nembutal i.p. (50 mg/100 g b.wt). The bones were defatted and dehydrated by 2 washes of chloroform: methanol (1 vol.: 1 vol.). They were then broken into 3–4 pieces each. From then on they were handled under aseptic conditions, since any

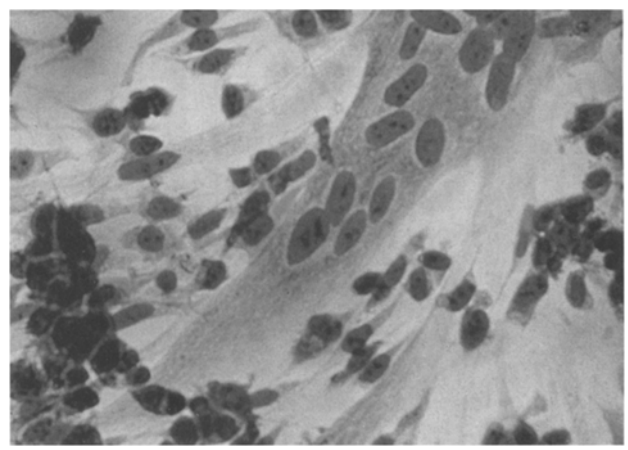

Figure 1. L6 myoblastic cells, aspect after short trypsinization as described in the text: partial disruption of the upper mononucleated cell sheet leaves the polynucleated myotubes intact. Fixation in methanol, staining by May-Grunwald-Giemsa[10].