Cell-type differences in the serum and cyclic AMP-dependent regulation of ornithine decarboxylase activity in two cultured fibroblast types

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Serum re-feeding stimulated ornithine decarboxylase (ODC) activity 8 to 10-fold in FS fibroblasts and 5 to 8-fold in 3T3 fibroblasts. Addition of dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine at the time of serum re-feeding further stimulated ODC activity in 3T3 fibroblasts but inhibited the serum stimulation of ODC activity in FS fibroblasts. It is suggested that serum and cyclic AMP independently regulate ODC activity in cultured fibroblasts.

Ornithine decarboxylase activity can be markedly increased by effectors of adenylate cyclase and cyclic AMP-dependent protein kinase (Jungman & Russell, 1977; Bachrach *et al.*, 1978). The role of cyclic AMP in the serum stimulation of ODC activity in fibroblasts, however, is still uncertain. Serum re-feeding of subconfluent fibroblasts stimulates ODC activity and synthesis (Lembach, 1974) but clearly decreases intracellular cyclic AMP concentrations (Pastan *et al.*, 1975). Conversely agents that increase cyclic AMP concentration, such as the phosphodiesterase inhibitor IBMX, increase ODC activity in cultured fibroblasts (Boucek & Lembach, 1977; Costa, 1978). IBMX probably increases ODC synthesis by an increase in cyclic AMP concentration and cyclic AMP-dependent protein kinase activity (Bachrach *et al.*, 1978; Russell & Haddox, 1979). On the basis of these reports it appeared that both serum and IBMX increased ODC activity, and probably its synthesis, but by different mechanisms. To determine if the serum and IBMX-mediated stimulation of ODC share a common cellular mechanism the effects of cyclic AMP effectors and serum re-feeding on ODC activity and cyclic AMP concentration were compared in FS and mouse 3T3 fibroblasts.

Materials and methods

The 3T3 mouse fibroblast line and FS fibroblasts were cultured by using routine sterile techniques.

Abbreviations used: ODC, ornithine decarboxylase; IBMX, 3-isobutyl-1-methylxanthine, FS fibroblasts, human foreskin fibroblasts.

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The cells were grown in sterile plastic culture flasks (25 cm² from either Corning Co. or Falcon Co.) at 37°C in Dulbecco's medium containing 10% (v/v) serum by the methods described by Lembach (1974). Powdered Dulbecco's medium (GIBCO) was reconstituted with double-glass-distilled water to which was added: Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (Sigma) to a final concentration of 20 mM and adjusted to pH 7.4; NaCl (1.6 g/litre); chlortetracycline hydrochloride (5 mg/ml; GIBCO); and NaHCO₃ (0.5 g/litre). The medium was sterilized by Millipore membrane filtration (0.2 µm). The FS fibroblasts were cultured in media containing foetal calf serum (GIBCO) and the 3T3 fibroblasts were cultured in media containing calf serum (GIBCO). Routine checks for sterility were performed and prolonged periods of confluency were avoided.

To ensure uniform growth characteristics in all experimental flasks for each protocol, a common stock culture was used to inoculate all flasks simultaneously at a density of 1.8×10^4 cells/cm². After plating, a recovery period of 24 h in Dulbecco's medium plus 10% serum preceded any experimental protocol. During this 24 h the cells attached and usually doubled as determined by microscopic examination and cell counting. After 24 h, the cell monolayer was washed with pre-warmed phosphate-buffered saline and overlaid with fresh Dulbecco's medium containing 0.5% serum for 18 to 24 h. The cell monolayers in 0.5% serum containing media were considered serum-deprived or 'quiescent' cultures. Serum re-feeding consisted of replacing the 0.5% serum-containing Dulbecco's medium with 10% serum-containing Dulbecco's

medium. In some experiments, the cell monolayer was maintained in medium containing 10% serum and was considered 'non-quiescent'.

At the appropriate time after serum re-feeding, the flasks were washed three times with phosphate-buffered iso-osmotic saline. The cell monolayer was overlaid for 5 min at room temperature with 0.5 ml of buffer consisting of: Tris/HCl (25 mM, pH 7.2), EDTA (0.1 mM), dithiothreitol (5 mM) and Triton X-100 (0.5%, v/v). The soluble phase was removed from the flask and centrifuged at 4°C in disposable Eppendorf tubes for 2 min at 8000g (Brinkman 3200). ODC activity was assayed in the supernatant fraction by a previously described method (Lembach, 1974). The recovery of ODC activity after Triton extraction was comparable with the recovery of ODC activity from a cell homogenate (R. J. Boucek, J. Weiss & K. J. Lembach, unpublished work). The final concentrations in the assay were 0.12 mM for pyridoxal 5'-phosphate and 1.0 mM for ornithine (sp. radioactivity of 2.1 $\mu\text{Ci}/\mu\text{mol}$).

Protein was measured in each assay tube as binding of Coomassie Blue and compared with a bovine serum albumin standard solution (Bradford, 1976). Enzyme activity was expressed as nmol of CO_2 liberated/mg of protein per h at 37°C.

Acid-extractable cyclic AMP content was assayed by the radioimmunoassay method of Harper & Brooker (1975). Rabbit antibody to thyroglobulin-linked-*O*-succinyl cyclic AMP for the cyclic AMP assay was provided by Dr. David Garbers, Department of Physiology, Vanderbilt University School of Medicine.

Results and discussion

Serum re-feeding of subconfluent 'quiescent' 3T3 mouse fibroblasts significantly increased the ODC specific activity. At 4–6 h after serum re-feeding there was an 8–10-fold increase in ODC specific activity that fell below the basal activity by 12 h after re-feeding. ODC specific activity in serum re-fed 3T3 fibroblasts was 10-fold higher than in serum re-fed FS fibroblasts. The addition of IBMX (1 M) at the time of serum re-feeding increased by 9-fold the ODC specific activity observed after re-feeding alone in the 3T3 fibroblasts. IBMX increased ODC activity in serum-re-fed 3T3 fibroblasts in a dose-dependent manner (Fig. 1). At 6–8 h after serum re-feeding of subconfluent 'quiescent' FS fibroblasts ODC specific activity increased 5–8-fold. The addition of IBMX at the time of serum re-feeding either had no effect or decreased the ODC specific activity observed after serum re-feeding alone in the FS fibroblasts (Fig. 1). ODC specific activity in serum-re-fed FS fibroblasts was not changed by the addition of low concentrations of IBMX (1 μM) but was inhibited at concentrations of IBMX greater than (10 μM) (Fig. 1).

Fig. 1. Effects of serum re-feeding with the addition of either IBMX or dibutyryl cyclic AMP on ODC activity. 'Quiescent' 3T3 ($\triangle$ and $\square$) or FS ($\blacktriangle$ and $\blacksquare$) fibroblasts were re-fed 10% serum containing Dulbecco's media. IBMX ($\triangle$ and $\blacktriangle$) or dibutyryl cyclic AMP ($\square$ and $\blacksquare$), at the indicated final molar concentration, was added at the time of serum re-feeding. ODC activity was determined at 4.5 h (3T3) or 6 h (FS) after serum re-feeding. Mean activities of duplicate determinations are shown either after serum re-feeding alone or after serum re-feeding plus the addition of either IBMX or dibutyryl cyclic AMP.

The addition of dibutyryl cyclic AMP at the time of serum re-feeding had effects comparable with IBMX on ODC activity in 3T3 and FS fibroblasts. Increasing the concentration of dibutyryl cyclic AMP at the time of serum re-feeding from 0.1 mM to 10 mM increased ODC specific activity by 5-fold in the 3T3 fibroblasts. Conversely increasing the concentration of dibutyryl cyclic AMP at the time of serum re-feeding from 50 μ M to 5 mM decreased ODC specific activity almost 5-fold in the FS fibroblasts.

The half-life of ODC activity was estimated in serum-re-fed and IBMX-treated 3T3 fibroblasts by the addition of cycloheximide (50 μ g/ml) 3 h after serum re-feeding with or without IBMX (1 mM). ODC activity was determined every half-hour for 3 h. By this method the half-life for serum re-feeding alone was 128 min, whereas for serum re-feeding in the presence of IBMX it was 115 min. IBMX probably increased ODC synthesis in 3T3 fibroblasts on the basis of the following observations. (a) IBMX increased ODC activity without an increase in half-life of ODC activity. (b) The addition of actinomycin D (4 μ g/ml) completely blocked the increase in ODC activity observed after the addition of IBMX alone to non-quiescent cells and an increase was observed after the addition of IBMX at the time of serum re-feeding.

Although other investigators have reported an inhibition of ODC activity by dibutyryl cyclic AMP in other cell types and/or growth states (Russell & Haddox, 1979; Otani *et al.*, 1980), the cellular mechanism of this inhibition remains uncertain. Possible explanations for a qualitatively different response of ODC activity to IBMX and dibutyryl cyclic AMP between the two fibroblastic cell types included: (a) the absence of cyclic AMP-dependent regulation of ODC synthesis in FS fibroblasts or the type I cyclic AMP-dependent protein kinase; (b) the lack of an increase in cyclic AMP concentration by phosphodiesterase inhibition in the FS fibroblasts; and (c) differences in the cell cycles or growth rate at the time of addition of IBMX (Kram *et al.*, 1973; Cress & Gerner, 1980).

We examined different serum concentrations and growth rates as possible determinants for the differential effects of IBMX on ODC activity between the two cell types. First, the addition of IBMX (1 or 0.1 mM) in the presence of either 10% serum ('non-quiescent') or 0.5% serum ('quiescent') was studied in both cell types. Both 3T3 and FS fibroblasts, cultured in medium containing 0.5% serum, had lower ODC specific activity and growth rates. The addition of IBMX to 'quiescent' 3T3 fibroblasts that were not serum re-fed increased ODC specific activity 10-fold compared with matched control cultures with no added IBMX. In contrast, the addition of IBMX (1 mM) to 'quiescent'

FS fibroblasts that were not serum re-fed inhibited ODC specific activity. Second, the addition of 1 mM-IBMX to 'non-quiescent' 3T3 fibroblasts in medium containing 10% serum also significantly increased ODC specific activity. Therefore, neither serum re-feeding nor the serum concentration was essential for IBMX to increase ODC activity in 3T3 fibroblasts, since IBMX increased ODC activity in both 'non-quiescent' cells in 10% calf serum and in 'quiescent' cells in 0.5% calf serum. Conversely, even in 'quiescent' cultures of FS fibroblasts, with low ODC activity that was clearly inducible by serum re-feeding, IBMX inhibited ODC specific activity at higher IBMX concentrations.

Because the transcriptional event in ODC regulation with serum re-feeding in 3T3 fibroblasts (Clark, 1974) and serum refeeding plus dibutyryl cyclic AMP in CHO cells (Costa, 1978) occurs within 1 h after their addition we determined the cyclic AMP concentrations 5, 10, 20, 30, 40, 50 and 60 min after the addition of serum to 'quiescent' FS fibroblasts. The cellular cyclic AMP content increased 3-fold within 5 min after the addition of IBMX and serum re-feeding in FS fibroblasts, whereas serum re-feeding alone resulted in a small decrease in cyclic AMP concentrations (results not shown). A similar increase in cyclic AMP content after IBMX addition and decrease in cyclic AMP content after serum re-feeding alone was observed in the 3T3 fibroblasts (results not shown) as has been reported by other investigators (Pastan *et al.*, 1975).

The increase in cyclic AMP in FS fibroblasts within the first hour after addition of IBMX indicated inhibition of phosphodiesterase by IBMX. Furthermore, dibutyryl cyclic AMP also inhibited the increase in ODC activity observed after serum re-feeding of FS fibroblasts. These observations would exclude possible cell-to-cell differences in the inhibition of phosphodiesterase by exogenously added IBMX as a likely explanation for the lack of stimulation of FS-fibroblast ODC activity by IBMX.

The amount and/or degree of activation of type I cyclic AMP-dependent protein kinase has been suggested to be important in the cyclic AMP-dependent regulation of ODC activity (Russell & Haddox, 1979). A predominance of type I protein kinase in 3T3, and its absence in FS, fibroblasts, would be predicted, although the inhibition of ODC activity in FS fibroblasts by concentrations of IBMX that increase ODC activity in 3T3 fibroblasts may be difficult to explain by this hypothesis.

Furthermore, only type II protein kinase was reported to be present in density-inhibited 3T3 cell lines (Gharrett *et al.*, 1976). Both types I and II protein kinases have been reported in the diploid human fibroblasts WI-38, and both type I and type II enzymes were activatable by increased cyclic AMP concentrations (Ray *et al.*, 1979).

Our results suggest that serum stimulation of ODC synthesis in fibroblasts may be independent of cyclic AMP content, similar to the cyclic AMP-independent stimulation of hepatic ODC by triiodothyronine (Combest & Russell, 1980). In 3T3 fibroblasts ODC synthesis may also be stimulated by a cyclic AMP-dependent mechanism, which was not demonstrable in the foreskin fibroblasts. This hypothesis is supported by the observations of Hogan *et al.* (1974), that serum stimulation of ODC activity in baby-hamster kidney fibroblasts may not be dependent on changes in cyclic AMP content. Therefore, the putative cyclic AMP-dependent mechanism that regulates ODC synthesis may vary from cell type to cell type because of: (a) an absence of a key element in the mechanism or modification of the mechanism (Clo *et al.*, 1979); (b) differences in compartmentalization of the phosphorylation system (Corbin *et al.*, 1977); or (c) a modification of the effect of cyclic AMP secondary to the type or degree of activation of cyclic AMP-dependent protein kinase (Byus *et al.*, 1978; Russell & Haddox, 1979). The heteroploid 3T3 mouse fibroblast cell line and the diploid (low-passage) FS fibroblasts have been shown to differ in the cyclic AMP-dependent regulation of ODC synthesis, which may have important implications in their growth regulation.

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