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ACTH-INDUCED INTERNALIZATION OF PLASMA MEMBRANE IN XANTHOPHORES OF THE GOLDFISH, CARASSIUS AURATUS L.

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Summary: The ACTH-induced pigment dispersion in primary cultures and in suspensions of xanthophores of the goldfish, Carassius auratus L., has been shown to include the formation of rosette-like membranous structures and plasma membrane invaginations (pits). The rosettes and pits are probably similar structures sectioned at different angles. Horseradish peroxidase studies demonstrate that these structures are eventually converted into small spherical vesicles and long smooth elements, similar in appearance to spherical and "tubular" endoplasmic reticulum, respectively. In addition, these studies show that once vesicles are formed they are no longer continuous to the outside of the cell.

INTRODUCTION

The classical concept of the action of most polypeptide hormones involves binding to the outside of plasma membrane via specific hormone receptors. This is followed by the action of an intracellular second messenger such as c-AMP or Ca^{++} while the hormone remains outside of the cell. Recent studies, however, indicate that in a number of systems the hormone-receptor complex is "internalized" (1-4). We wish to report here that in the case of goldfish xanthophores, the hormone ACTH induces plasma membrane internalization via the formation of pits and the generation of endocytotic vesicles near the bottom of the pit, eventually producing intracellular membranous organelles resembling smooth endoplasmic reticulum.

METHODS

Cell Isolation and Culture. Xanthophores were isolated from fins of xanthic goldfish by treating with 0.02% EDTA (footnote 1) and 0.25% trypsin in

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Abbreviations: EDTA-ethylene diaminetetracetic acid; ER-endoplasmic reticulum; HRP-horseradish peroxidase.

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a Ca- and Mg-free phosphate buffer (0.1 M, pH 7.2). The cells were collected by centrifugating at 1000 Xg for 5 min and then resuspended in a growth medium (80% medium 199 and 20% fetal calf serum). Some of these cells were cultured as described elsewhere (5)

Cytochemistry. Cell suspensions were incubated with 0.2 I.U./ml ACTH and 5 mg/ml horseradish peroxidase (HRP) for 10 min, 1 hr and 2 hr. Other cells treated with ACTH for 50 min were followed by a 10 min incubation in ACTH plus HRP. Control cells were incubated with HRP without ACTH. Cell pellets were fixed with 1.25% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 1 hr, washed with buffer twice and then incubated with 10 mM 3,3'- diaminobenzidine tetrahydrochloride (Sigma Chem, Co, St. Louis, Mo.), 0.015% H_2O_2, and 5 mM KCN in 0.05M tris-HCl buffer (pH. 7.0) for 1 hr at 37°C in the dark (6). Control experiments included cells incubated in HRP but without ACTH and cells incubated in ACTH and HRP without subsequent cytochemical development. Following incubation, cells were washed twice in 0.1 M cacodylate buffer and then prepared for transmission electron microscopy.

Electron Microscopy. For scanning electron microscopy, cell suspensions with or without ACTH (0.2 I.U./ml), were placed on plastic slides for 1 hr to allow loose attachment of cells. After 3% glutaraldehyde fixation in 0.1 M cacodylate buffer (pH 7.4), xanthophore locations were marked and photographed. They were then post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) followed by dehydration in an ethanol series. Finally they were dried in a critical point apparatus, sputtered with gold and observed in a JOEL JSM-2 scanning electon microscope. For transmission electron microscopy, suspensions or cultures of attached cells were fixed at room temperature with 3% glutaraldehyde and 1% OsO_4, both in 0.1 M cacodylate buffer (pH 7.4) for 1 hr respectively. After a graded ethanol dehydration, cells were embedded in Epon 812, and the sections were examined unstained or stained with uranyl acetate and lead citrate with a Philips 201 or 301 electron microscope at 80 kV.

RESULTS

Xanthophores, in suspension or in culture, showed the following consistent ultrastructural differences with and without ACTH treatment: (i) ACTH-treated cultured xanthophores show frequent rosette-like membranous structures (see insert, Fig. 1a), occasionally plasma membrane invaginations (pits) and large intracellular membranous structures with associated small vesicles (Fig. 1a and 3a). The rosettes and the pits are probably similar structures sectioned at different angles. When cultured xanthophores are sectioned horizontally, rosettes are encountered far more often than pits which tend to be perpendicular to the plasma membrane (Fig. 3a). Rosettes are rarely encountered in cultured xanthophores without ACTH treatment. (ii) ACTH-treated xanthophores in suspension show smooth ER-like structures surrounding single or multiple pterinosomes, the cell's pigmentary organelles. These consist of pairs of membranes 50 to 60 nm apart and often exhibit small membranous buds

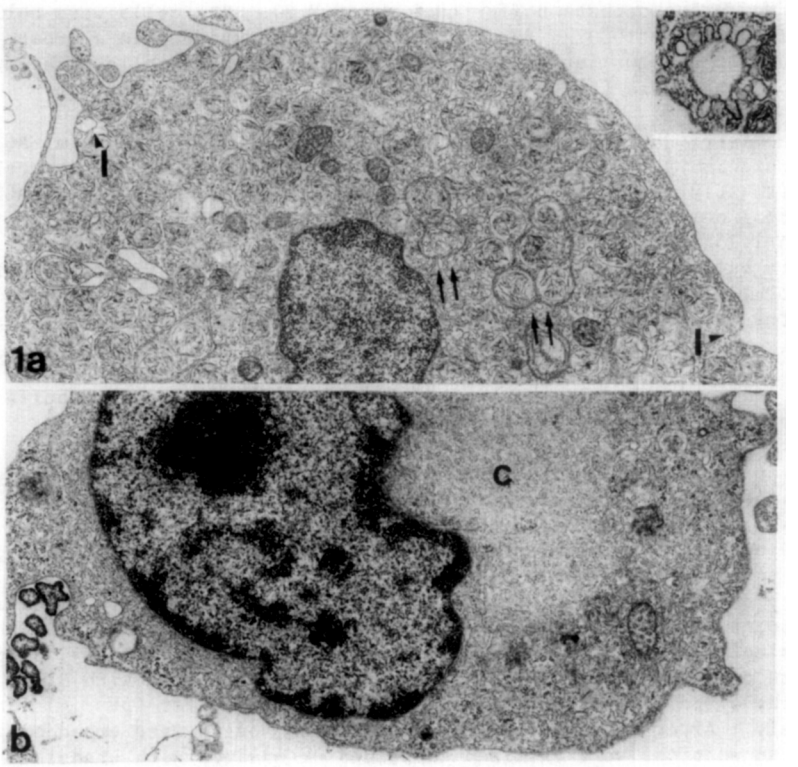

Fig. 1 Xanthophores in suspension a) ACTH-treated cell with many pterinosomes surrounded by smooth ER-like structures with budding vesicles (arrows). There are several small invaginations (I) of the plasma membrane. Horizontal sections of cells show many rosette-like structures (insert) x 8,200, insert x 19,100. b) In cells not treated with ACTH these structures are absent or very rare. Note the large aggregate of carotenoid droplets-smooth ER (c) in these cells. x 11,000.

(Fig. 1a). Such structures are absent, or very rare, in non- ACTH-treated cells (Fig. 1b). (iii) Scanning electron microscopy showed a large number of pits (plasma membrane invaginations) in ACTH-treated xanthophores in culture (Fig. 2a) or in suspension (Fig. 2b). Such pits are rare in non-ACTH- treated xanthophores in culture (Fig. 2c) or in suspension (Fig. 2d). (iv) When xanthophores in suspension were incubated with HRP and ACTH, striking internalization of HRP was observed. After 10 minutes of incubation, HRP-positive vesicles were seen near the plasma membrane (Fig. 3a). Some of these vesicles show HRP-positive buds. After 1 to 2 hours of incubation,

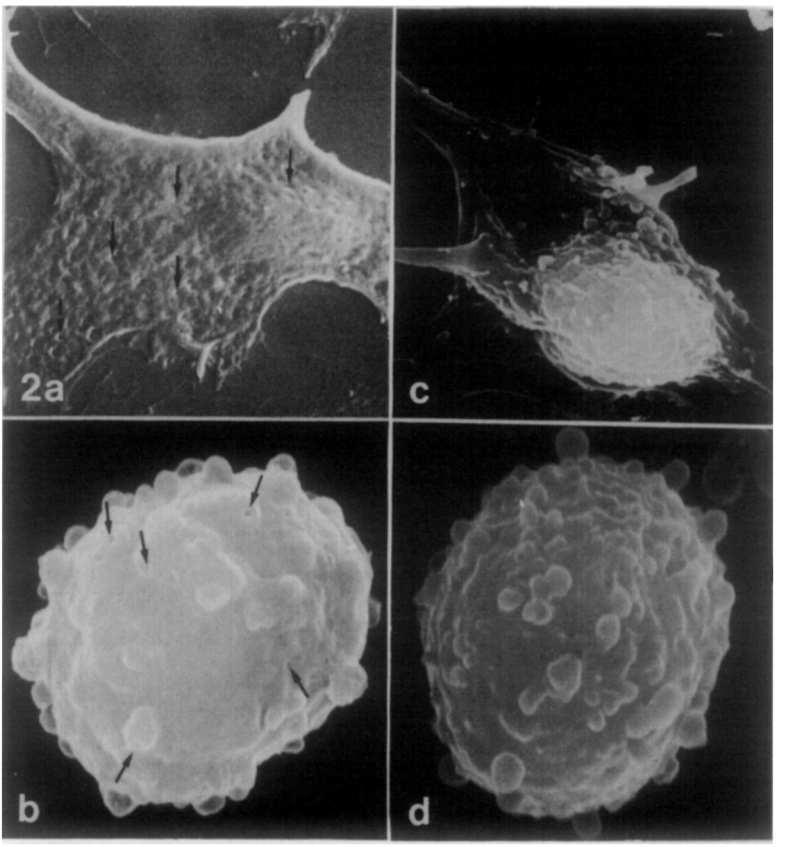

Fig. 2 Scanning electron micrographs of xanthophores in culture (a and c) and in suspension (b and d). The ACTH treated cells (a and b) have large numbers of pits (arrows) on the surface while the control cells (c and d) have few or no pits. 2a,c x 4,200, 2b,d x 7,200.

HRP-positive structures were present throughout the cell. These include rosette-like structures and "tubular elements", often surrounding pterinosomes and with membranous buds. The results show that these structures, observed initially in the absence of HRP (Fig. 1a), are derived from plasma membrane invagination (Fig. 3b). Suspended xanthophores treated with ACTH for 50 min. followed by a 10 min incubation with HRP and ACTH reveal that most of the membranous structures in the cell are HRP-negative, with only a few structures, usually round and near the periphery, being positive. This indicates that after 50 minutes of incubation, the internal vesicles are no longer continuous

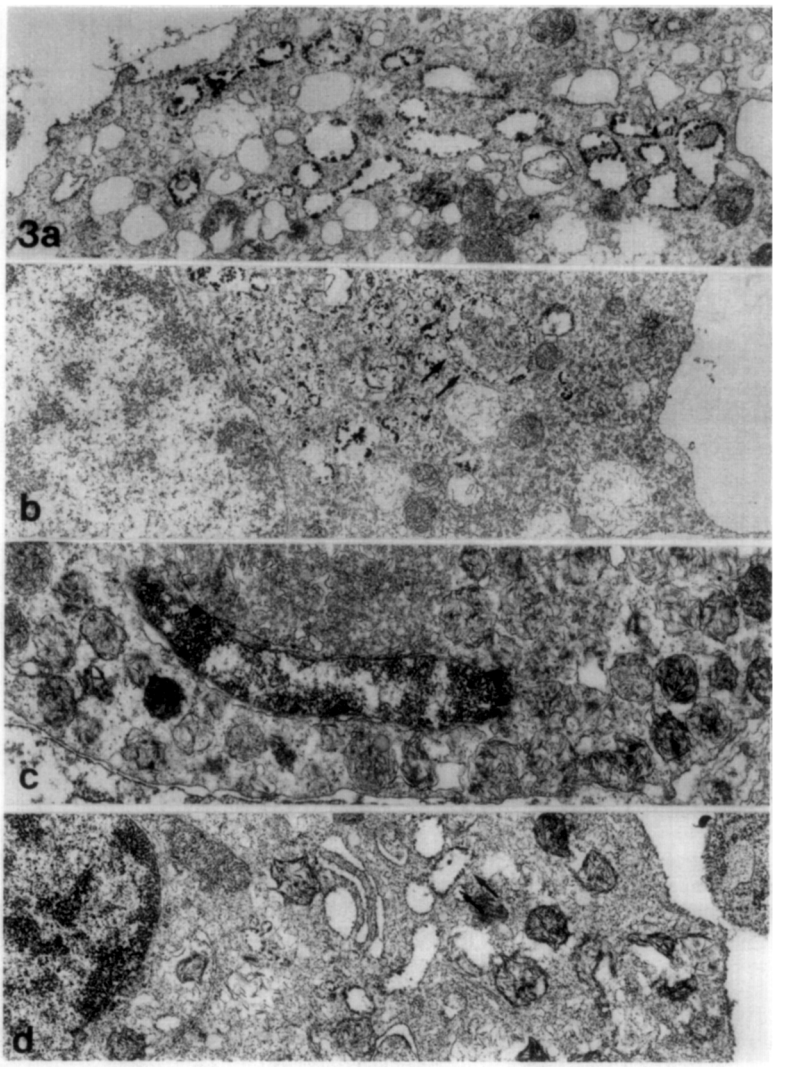

Fig. 3 Demonstration of ACTH-induced plasma membrane internalization by horseradish peroxidase. Xanthophores in suspension were treated with ACTH and HRP for 10 min. (a) or 1 hr. (b). Within 10 min., large HRP-positive vesicles are seen, primarily near the plasma membrane. After 1 hr., elongated ER-like HRP-positive structures, often with budding vesicles, are present throughout the cell. Some of these surround pterinosomes (arrows). Control xanthophore (c), incubated for 1 hr. with HRP but no ACTH, show no HRP-positive structures. When xanthophores were treated with ACTH for 50 min, followed by 10 min with ACTH and HRP (d), most of the membranous structures in the cell are HRP-negative. Only some round vesicles (arrows), usually near the plasma membrane, are HRP-positive. Magnification: 3a x 14,900, 3b x 16,200, 3c x 17,800, 3d x 14,200.

to the outside (Fig. 3d). In contrast to ACTH-treated cells, xanthophores in suspension that were not treated with ACTH showed little or no HRP internalization (Fig. 3c).

DISCUSSION

In the recent past, a number of laboratories have reported that large polypeptides, including several hormones, are "internalized" by target cells. This appears to be via binding to specific receptors, followed by internalization of the plasma membrane. The ultimate intracellular site of delivery, however, varies from one system to another (4,7,8,). MSH has been reported to be delivered in a certain melanoma cell line to the Golgi complex and/or pigment organelles (melanosomes) to produce c-AMP *in situ* which, perhaps, would activate latent tyrosinase (9).

The present study demonstrates that ACTH stimulates goldfish xanthophores to internalize their plasma membrane which is eventually converted to two types of structures: small spherical vesicles and long smooth elements, similar in appearance to spherical and "tubular" endoplasmic reticulum (Fig. 3b). The latter are probably cross-sections of flattened sacs, which often surround one or more pterinosomes whereas the spherical vesicles are scattered throughout the cytoplasm. This pattern in quite different from the examples of protein internalization cited earlier, but is very similar to the plasma membrane internalization observed in macrophages (10). In these cells, stereological studies showed that the amount of membrane internalized is approximately 300% per day, only a small portion of which ends up in lysosomes. It was, therefore, concluded that most of the internalized plasma membrane must be re-utilized for plasma membrane formation (11). In the xanthophores, there appears to be very few lysosomes and the extent of plasma membrane internalization, by comparison of transmission electron microscopic images, is similar to that in macrophages. Clearly, it appears that most of the internalized plasma membrane must be re-utilized for plasma membrane formation by process(es) as yet unknown. The relationship between this hormone-induced

plasma membrane internalization and the physiological effects exerted by ACTH on the cells (5,12) are currently under investigation.

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