Biochemical and cytochemical studies on adenylate cyclase activity in the developing rat submandibular gland: differentiation of the acinar secretory compartment

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SUMMARY

To investigate membrane changes in development of the exocrine cells of the rat submandibular gland (SMG), biochemical and cytochemical studies of adenylate cyclase activity were performed on prenatal and postnatal glands. SMG rudiments and glands were studied from 15 days of gestation up to birth and 1, 2, 3, 4 and 24 weeks after birth. Glands were chemically assayed for adenylate cyclase activity using the procedures of Salomon and co-workers and cytochemically studied using a procedure which was verified biochemically. At 15–16 days of gestation basal adenylate cyclase activity was low and no staining could be observed. Adenylate cyclase activity rose six-fold from the 16th to the 18th day of gestation. Adenylate cyclase staining became evident along the surface of most of the cells of the rudiment at this time. Basal adenylate cyclase activity remained relatively constant from the 18th day of gestation up to 24 weeks of age. However, sequential changes were seen in the cytochemical localization, especially in relation to the apical plasma membrane of the developing secretory cells.

INTRODUCTION

During the cytodifferentiation of the acinar exocrine cells of the rat submandibular gland (SMG), the cells show an orderly sequence of appearance of the various organelles required for the synthesis and packaging of their secretory product. Within the cytoplasm, there is an apical-basal polarization of cellular organelles (Cutler & Chaudhry, 1974). This orderly appearance and organization of organelles seems to be initiated on the 16th day of gestation in the undifferentiated epithelial cells of the end-buds of the early arborizing SMG rudiment. Following direct interaction between the end-bud epithelial cells and the surrounding mesenchyme, the end-bud cells begin to alter their organelle pattern and initiate synthesis of early secretory product (Cutler & Chaudhry, 1973a; Yamashina & Barka, 1973). Once the basic topographic organization of organelles has been established within the developing secretory cells, there is a

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period during which the cells accumulate rough endoplasmic reticulum (r.e.r.) and synthesize increasing amounts of secretory product.

Cyclic nucleotides, specifically adenosine 3',5' cyclic monophosphate (cyclic AMP, cAMP), may be determinants of cellular differentiation (see McMahon, 1974, for review). Elevated intracellular levels of cyclic AMP seem to stimulate production of cell-specific proteins and enhance the stability and assembly of some organelles. There also appears to be an inverse relationship between cell division and cAMP levels such that increased intracellular cyclic AMP inhibits cell division and DNA synthesis. An inverse relationship between increasing levels of cellular differentiation and cell division has been noted in the developing rat SMG (Cutler & Chaudhry, 1974) and various other exocrine glands (Redman & Sreebny, 1970; Pictet, Clark, Williams & Rutter, 1972).

The current report concerns adenylate cyclase in the developing rat submandibular gland.

METHODS AND MATERIALS

Female Sprague-Dawley rats (Charles River) were bred under rigidly controlled procedures (Cutler & Chaudhry, 1975). The criteria for insemination was the observation of sperm on vaginal cytology following mating. Zygotes were considered to be zero-hours-old at 8.00 a.m. on the day sperm was found (Cutler & Chaudhry, 1973b).

Adenylate cyclase assay of the developing rat SMG

SMG rudiments and glands were examined from the 15th day of gestation until birth (21.5 days of gestation ± 4 h) and 1, 2, 3, 4, and 24 weeks after birth. Glands were pooled as necessary to give adequate protein concentrations for the assay. In all cases the glands or rudiments were homogenized in a glass tissue grinder in 0.01 M Tris buffer (pH 7.4) and then assayed for basal adenylate cyclase activity using the methods of Salomon, Londos & Rodbell (1974). An aliquot of the homogenate (approximately 100 μg of protein as determined by Lowry protein assay (Lowry, Rosebrough, Farr and Randall, 1951) was incubated for 15 min in a 100 μl of assay mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM-MgCl₂, 1 mM cAMP, 1 mM dithiothreitol, 10 mM phosphocreatine, 50 units/ml phosphocreatine kinase and 0.1 mM ATP (3 x 10⁶ cpm α-³²P-ATP). The incubation was stopped by adding 100 μl of stopping solution (4 mM ATP, 1.4 mM cAMP and 2 % sodium dodecyl sulfate) and boiling for 3 min; 20000 cpm of [³H]cAMP were then added for estimation of the recovery from chromatography on Dowex Ag50W-X4 (200–400 mesh) and neutral alumina columns. The eluates were collected in scintillation vials containing Bray's solution (Bray, 1960) and duplicate samples were counted for 10 min in a liquid scintillation counter set with separate channels for [³H] and ³²P. The results are expressed in picomoles cAMP produced per milligram protein per 15 min.
Adenylate cyclase in developing salivary glands

SMG rudiments from three litters (at least 24 fetuses) were examined at each prenatal period. Glands from 12 animals from three different litters were studied at each postnatal time point. The glands or rudiments were excised and immediately minced into small cubes (> 1 mm³) and fixed by immersion in 1.25 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 4 °C. The tissue was then washed 4 times (30 min each) in cold 0.1 M cacodylate buffer (pH 7.3) and each block was cut into thin slices using a razor blade and a dissecting microscope.

Aliquots were incubated in a modified Howell & Whitfield media (1972): 80 mM Tris-maleate buffer (pH 7.4) with 8 % sucrose (w/v), 3 mM lead nitrate, 10 mM or 20 mM sodium fluoride, 4 mM magnesium sulfate, 2 mM theophylline and 1 mM 5'-adenylyl-imidodiphosphate (AMP-P(NH)P). AMP-P(NH)P is a specific substrate for adenylate cyclase. The incubation was carried out at 30 °C with a gentle agitation for 45 min. The reaction was stopped by removing the medium, quickly washing the tissue twice in 0.1 M cacodylate buffer (pH 7.3) and then post-fixing the tissues in 1 % osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 1 h. The tissues were then washed with distilled water, dehydrated through a series of ethanols and propylene oxide and embedded in Epon 812.

Control specimens included heat-activated tissue and tissue incubated in media without substrate.

Biochemical verification of adenylate cyclase cytochemical procedures

Tissue was either fixed in 1.25 % glutaraldehyde, washed in 0.1 M cacodylate buffer (as described above) and homogenized in 0.01 M Tris buffer (pH 7.4) in a glass tissue grinder, or the tissue was first homogenized and then the homogenate was fixed for 15–30 minutes in 1.25 % glutaraldehyde and washed in 0.1 M cacodylate buffer (pH 7.4). Adenylate cyclase activity (basal and fluoride-stimulated) was measured in fresh and fixed tissues using the method of Salomon et al. (1974), as described above. The results are reported in picomoles of cAMP produced per milligram of protein in 15 min.

RESULTS

Basal adenylate cyclase activity in the developing SMG

The levels of adenylate cyclase activity in the prenatal and post-natal SMG are shown in Fig. 1. Basal adenylate cyclase activity was very low at 15 days of gestation, but had increased six-fold by the 18th day of gestation, after which it stayed essentially constant. The basal activity seen in the fully matured adult gland was the same as that seen in an 18-day prenatal rudiment. There was a
The adenylate cyclase activity of the rat SMG during prenatal and postnatal development (Mean ± standard error).

25–30% drop in basal adenylate cyclase activity 1–2 weeks after birth and then specific activity returned to the levels seen at birth.

**Cytochemical studies on adenylate cyclase activity in the developing SMG**

No cytochemical evidence of adenylate cyclase activity was noted in 15-day or 16-day SMG rudiments whether 10 mM or 20 mM NaF was used in the assay media and no reaction product was seen at sites of epithelial-mesenchymal or epithelial-nerve contact in 16-day rudiments (Fig. 2).

On the 17th day of gestation, reaction product indicative of adenylate cyclase activity became apparent at the plasma membrane of several cells within the

**Figures 2 and 3**

Fig. 2. Electron micrograph showing portions of cells from an end-bud of the SMG rudiment (SMG) at 16 days in utero. An epithelial cell process (Box) penetrates the basal lamina (bl) to form a direct contact with a neighbouring mesenchymal cell (MES) (× 25000). Inset: Higher magnification electron micrograph of the epithelial mesenchymal contact in the box (×80000). No reaction product was found. Section counter-stained with uranyl acetate and lead citrate.

Fig. 3. Electron micrograph showing reaction product indicative of adenylate cyclase activity (arrows) in association with the cell surface of end-bud cells from a 17-day embryonic SMG rudiment. Tissue incubated in media containing 10 mM NaF (× 25000). Section counter stained with uranyl acetate and lead citrate.
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end-buds of the differentiating rudiment (Fig. 3). Late on the 17th day of gestation significantly more cells showed surface reaction product, associated with the plasma membrane adjacent to newly forming lumina of the developing terminal tubules. Some single tubule cells had dense reaction product associated with their luminal microvilli but little or none at their lateral surface (Fig. 4). Early on the 18th day of gestation all the cells surrounding the lumina of the more advanced tubules demonstrated reaction product along their luminal plasma membrane (Fig. 5). This membrane-associated reaction product was often observed in small subsurface vesicles that appeared to be opening onto the plasma membrane (Figs. 5 & 6). Occasionally, small vesicles at the distal ends of Golgi profiles contained reaction product.

Secretory granules first appeared in cells on the 18th day of gestation. Cells which contained these osmophilic, electron dense (type I) granules did not have cytochemically demonstrable adenylate cyclase activity, while the neighboring cells without granules retained such activity (Fig. 7). This differential staining of terminal tubule cells was maintained throughout prenatal and postnatal development of the gland.

On the 20th day of gestation a lucent secretory granule (type II) was seen within some of the terminal tubule cells. Cells containing type II granules and cells without granules showed adenylate cyclase reaction product along their apical surfaces (Fig. 8), while cells containing type I granules were non-reactive for adenylate cyclase.

Cells containing type II granules were considered proacinar cells (Yamashina & Barka, 1973; Cutler & Chaudhry, 1974). These cells retained their apical adenylate cyclase reactivity as they matured (Fig. 9). At 2½ weeks after birth developing myoepithelial cells also showed adenylate cyclase reactivity (Fig. 10). However, upon reaching full maturity (about 12 weeks of age) both acinar cells and myoepithelial cells were virtually non-reactive for adenylate cyclase with

Figures 4-6

Fig. 4. Electron micrograph showing dense cyclase reaction product (arrows) deposition in association with the luminal microvilli of a terminal tubule cell from the SMG rudiment from a 17-day fetus. Very little reaction product is seen at the lateral surfaces of the same cell (× 25000). Section counter-stained with uranyl acetate and lead citrate.

Fig. 5. Electron micrograph showing the lumen (L) of a more advanced terminal tubule from the SMG of an 18-day fetus. The luminal plasma membrane of every cell lining the lumen shows reaction product. Several subsurface vesicles also show reaction product (arrows) (× 17000). Section counter-stained with uranyl acetate and lead citrate.

Fig. 6. Electron micrograph showing the apical surface of a terminal tubule cell from an 18-day embryonic SMG rudiment. A vesicle containing reaction product appears to be opening onto the cell surface (arrow) (× 64000). Section counter-stained with uranyl acetate and lead citrate.
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only small amounts of reaction product seen along intercellular secretory canaliculi. Control samples were always negative for lead deposits.

The results of the assay for adenylate cyclase in SMG tissue after fixation and with incubation in media containing 3 mM Pb (NO₃)₂ and the other constituents of the cytochemical procedure are shown in Table 1. Both embryonic and adult tissues retained about 15% of their basal activity under the conditions of assay. The remaining activity could be stimulated five- to six-fold by the addition of 20 mM NaF.

DISCUSSION

It has recently been postulated that cell differentiation may be controlled by changes in content of cyclic nucleotides, in particular cyclic AMP (McMahon, 1974). Cyclic AMP levels are in part controlled by adenylate cyclase. Intracellular cAMP levels could be elevated by synthesis of more adenylate cyclase. Such a mechanism has been demonstrated in several systems (Anderson & Pastan, 1975). Our results show that in the developing SMG rudiment adenylate cyclase activity rises sharply during early morphogenesis of the rudiment, over the short period of time when the differentiating secretory cells develop the structural configuration of typical exocrine cells. This period precedes the production of visible secretory granules and follows the establishment of the morphogenetic patterning of the gland, suggesting that differentiation of the developing exocrine cells may be associated with a greatly increased synthesis of cAMP. Similar observations have been made on developing fibroblasts in culture (Anderson & Pastan, 1975). Once definitive secretory cells appear, the overall cyclase activity remains relatively constant throughout further maturation of the gland.

Adenylate cyclase activity in the gland decreased 25–30% between 1 and 2 weeks after birth, as reported also by Barka and Van der Noen (1974). This fluctuation might be related to suckling or to early changes in ductal differentiation (Cutler & Chaudhry, 1975).

While the mean activity of adenylate cyclase remained relatively constant

Figures 7 and 8

Fig. 7. Electron micrograph showing the apical surface of several cells from a terminal tubule from the SMG rudiment of a 19-day embryo. The cells with type I granule (I) have no reaction product at their apical cell surface, while the cell without granules has reaction product (arrow) (× 10000).

Fig. 8. Electron micrograph showing the apical portions of several luminal cells from a terminal tubule of a 20-day embryonic rat SMG. Cells containing type I secretory granules (I) do not show reaction product deposition. However, cells which do not have secretory granules (arrow) or cells which contain type II secretory granules (II) show dense reaction product deposition along their apical plasma membranes. Golgi units (G) are prominent in several cells. There is a notable absence of reaction product at the lateral surfaces of the cells (× 12500).
Fig. 9. Electron micrograph showing the luminal area in developing acinus from a 2½-week-old rat. Reaction product is only seen in association with the luminal plasma membrane of cells containing more adult-type secretory granules (IV). The cell containing type I granules (I) is free of reaction product (× 25000).

Fig. 10. Low power electron micrograph showing periphery of a developing acinus from the SMG of a 2½-week-old rat. The tissue was incubated to show adenylate cyclase activity. Reaction product is seen in association with the more apical surface of the developing myoepithelial cells (MEC) (× 6250).
throughout development, there were marked changes in the cytochemical localization of adenylate cyclase reaction product. The precursor cells of the intercalated duct were negative for adenylate cyclase, while the proacinar cells had reaction product associated with their apical surface. Thus a correlation may exist between adenylate cyclase activity associated with the luminal membrane, and the final path of differentiation of the cell.

The reaction product for adenylate cyclase is associated with the apical (luminal) plasma membrane of the developing proacinar cells throughout glandular development, showing that there are regional differences in the composition of the membrane. Berridge, Lindley & Prince (1974) have suggested that cAMP is involved in the activation of an electrogenic pump associated with the apical plasma membrane during secretion by cells from the salivary glands of the blowfly. If such a mechanism is involved in the developing rat SMG, the localization of adenylate cyclase at the apical plasma membrane of the exocrine cells provides a convenient mechanism for the production of cAMP adjacent to its site of action.

Recently, there has been criticism of the use of lead nitrate in the cytochemical localization of adenylate cyclase (LeMay & Jarrett, 1975). The biochemical confirmation of the cytochemical procedures used in this study and in other studies on different tissues (Cutler, 1975) indicated that this procedure is useful in localizing adenylate cyclase activity.

Anderson & Pastan (1975) have demonstrated that in transformed cells the changes observed in cyclic AMP levels were partly modulated by alterations in adenylate cyclase activity. They also showed that the establishment of stability in contact-inhibited fibroblast populations was closely linked to increased adenylate cyclase activity, leading to elevated cyclic AMP levels in the cultures. Since differentiation results in the establishment of stability, our observations parallel

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Table. 1. *Adenylate cyclase activity of SMG tissues under conditions of the cytochemical assay*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pmole cAMP/mg protein/15 min</th>
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<tr>
<td></td>
<td>20-day embryonic SMG</td>
</tr>
<tr>
<td>SMG</td>
<td>65.1</td>
</tr>
<tr>
<td>SMG + 10 mM NaF</td>
<td>429.5</td>
</tr>
<tr>
<td>SMG (fixed)</td>
<td>10.6</td>
</tr>
<tr>
<td>SMG (fixed) + 10 mM NaF</td>
<td>28.5</td>
</tr>
<tr>
<td>SMG (fixed) + 3 mM Pb(NO$_3$)$_2$ + 10 mM NaF + 1 mM theophylline</td>
<td>10.9</td>
</tr>
<tr>
<td>SMG (fixed) + 3 mM Pb(NO$_3$)$_2$ + 20 mM NaF + 1 mM theophylline</td>
<td>56.4</td>
</tr>
<tr>
<td>SMG (fixed) (boiled) + 3 mM Pb(NO$_3$)$_2$ + 10 mM NaF + 1 mM theophylline</td>
<td>0</td>
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those seen in contact-inhibited fibroblasts. The cytochemical observations sug-
ggest that individual cells within a heterogenous population modulate their adeny-
late cyclase activity with regard both to amount and distribution along the cell
surface.

The observations presented in this study emphasize the dynamic changes which
occur at the cell surface during differentiation, while the rise in adenylate cyclase
activity observed during the early stages of secretory cell development suggest
that increasing levels of cyclic AMP are involved in the control of cellular dif-
ferentiation.

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