Stimulation of DNA Synthesis of Rat Salivary Gland Cells in Monolayer Cultures by Isoproterenol

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SUMMARY

Isoproterenol is a potent stimulator of DNA synthesis of salivary glands. The exact mechanism of its action is not clear. It is possible that isoproterenol may act directly upon salivary gland cells or that its effects may be mediated as a secondary phenomenon from prime effects on another tissue or organ system. In this report, evidence is presented that isoproterenol can directly stimulate DNA synthesis of cell cultures obtained from rat salivary glands.

INTRODUCTION

IPR is a $\beta$-adrenergic drug which when chronically administered in pharmacological doses can cause hyperplasia of the parotid and submaxillary glands of rats and mice (12). Within 20 hr after a single injection, DNA synthesis begins in the parenchymal cells of the salivary gland (2). This activity peaks at 28 hr and is followed by mitosis.

Macromolecular synthetic events preceding this wave of cellular proliferation have been well characterized (3, 10, 15). However, it is not yet clear whether the drug acts directly on glandular epithelial cells or whether the effect is secondary to the effects of IPR on other cells or organs.

Suggestive evidence of a direct effect has been obtained. IPR stimulation of DNA synthesis in salivary glands is unaffected by adrenalectomy (9), thyroidectomy (4), hypophysectomy (1), or even total denervation of the salivary gland (14). Further, after systemic injection, tritium-labeled IPR concentrates first in the nuclei of the salivary parenchymal cells (9). The stimulation is more intense if the drug is injected directly into the gland rather than administered systemically (9).

Despite these findings, there is no direct evidence that the drug has primary effects on the salivary gland. One approach would be to treat salivary gland cells in tissue culture with IPR and to measure the subsequent incorporation of TdR-3H into cellular DNA. Previous attempts with organ cultures in other laboratories have not been successful (9). Organ cultures of rat exocrine glands survive for only brief periods (8). Observations on the effects of IPR on monolayer cell cultures obtained from rat salivary glands are presented in this report. Specifically, attempts were made to establish cultures of salivary gland epithelial cells, demonstrate the presence of parenchymal cells by identifying specific synthetic products (sialomucin and amylase) of salivary epithelial cells in the cultures, determine the effects of IPR treatment on the incorporation of TdR-3H into DNA, and determine the optimal IPR dose for any observed stimulation.

MATERIALS AND METHODS

Cell Cultures. A variety of methods were used in attempts to establish cultures. The method which succeeded was as follows: A weanling Lewis rat (Microbiological Associates, Walkersville, Md.) was killed with ether. The submandibular and parotid glands were aseptically excised, minced with scissors, and placed with medium in a 6-oz prescription bottle. The initial medium was minimal essential medium with 10% dialyzed fetal calf serum supplemented with sodium pyruvate, nonessential amino acids, penicillin, and streptomycin. The cultures were not disturbed until the 4th day after planting, when islands of epithelial cells admixed with fibroblasts had migrated out of the tissue fragments adherent to the glass. When the monolayer became confluent, the cells were suspended with 0.25% trypsin and passaged routinely in 250-mi plastic flasks (Falcon Plastics, Los Angeles, Calif.), or 32-oz prescription bottles. By the 4th passage, or after approximately 8 weeks of in vitro culture, sufficient cells were available for experiments. All tests were performed on cells subcultured in 60-mm plastic Petri dishes. Monolayers for experiments were at least 1 week old and confluent. Cultures were exposed to IPR (Winthrop Laboratories, New York, N. Y.) which was freshly dissolved in trichloroacetic acid.

Amylase Assay. Amylase activity was assayed by the method of Caraway (6), based upon the decolorization of the starch-iodine reaction product.
Sialomucin Demonstration. The technique of Spicer et al. (13) was used, with minor modification. Cells cultured on coverslips were fixed in neutral-buffered formalin. Controls were digested for 18 hr at 36° with Vibrio cholerae neuraminidase (Calbiochem, Los Angeles, Calif.), 100 units/ml final concentration. The solution also contained 0.1 M sodium citrate and 0.04 M calcium chloride. Both control and test coverslips were stained, in triplicate, in 0.1% Alcian blue in 3% acetic acid, followed by 1% neutral red.

Assessment of DNA Synthesis. TdR-3H (NET-027; specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to cultures (1 μCi/ml) for specified periods. At the conclusion of the pulse, the medium was decanted and the monolayers were rinsed 3 times with cold 0.15 M NaCl solution. The cells were lysed with 3.0 ml 0.3 N KOH, collected in centrifuge tubes, and incubated overnight at room temperature to hydrolyze RNA. The DNA and protein was precipitated with cold 10% TCA and washed 3 times by centrifugation (2000 rpm, 30 min, 4° in a PR-2 centrifuge). At each washing, the precipitates were finely dispersed with a vortex mixer in 31 ml fresh TCA. The final pellet was redissolved in 0.3 N KOH. The DNA concentration was determined (5) with calf thymus DNA as the standard (Worthington Biochemicals Corp., Freehold, N. J.). An aliquot of 100 μl was placed on Whatman No. 3MM filter paper discs, dried, and counted in a Beckman LS-150 liquid scintillation counter, with an appropriate dilution in toluene of 25X scintillator (Catalog No. 6003004, Packard Instruments Co., Downers Grove, Ill.). Quenching and self-absorption could not be measured in this system, but there was a strict linear relationship between the amount of TdR-3H-incorporated cell fraction placed on the filter discs and the counts recorded.

Alternatively, in some experiments the method of Regan and Chu (11) was used. In this application, the initial KOH hydrolysate was placed directly on the filter discs. The discs were then dried, collected in a 1-liter Erlenmayer flask, and washed in 3 changes of 200 ml each cold 10% TCA, 3 changes of 95% ethanol, and an ether rinse. After the discs were completely dry, they were placed in scintillator, and each was counted for 10 min.

RESULTS

Presence of Salivary Gland Parenchymal Cells in the Cultures. Foci of polygonal cells could easily be found in early passage (Passages 4 to 8) cultures (Fig. 1). The numbers of these cells decreased with repeated subculture. Beyond 10 passages, epithelioid cells were not detectable.

Amylase activity was measured in the media of 4 cultures and in the homogenized cells of the monolayers. No significant difference in amylase activity was found between cultured medium, homogenized cells, or the controls which were aliquots of fresh medium from the same pool which had been exposed to the cells. It was concluded that amylase was not produced in the cultured cells.

Sialomucin was demonstrated in cultures. The following was interpreted as confirmation of its presence: (a) Alcian blue-staining fibrillar material at the immediate periphery of epithelioid cells (Fig. 1); (b) abolition of the positive staining reaction after 18 hr incubation with neuraminidase.

Measurement of DNA Synthesis. Six experiments were performed. Two experiments with 8th and 10th passage cells showed no significant difference between values obtained from control and IPR-treated (20 μg/ml) cultures. In the
remaining 4 experiments with cells from Passages 5 to 7, highly significant differences were obtained (Table 1).

In most experiments, total DNA was measured for each culture, but a significant increase in the total DNA of IPR-treated cultures was not detectable. There were no significant differences in the amount of DNA in different replicate cultures of any experimental group. Therefore, there was no advantage in determining the specific activity of the DNA of each culture.

**Optimal Dose of IPR.** The data from 3 separate experiments are presented in Chart 1. A dose of 50 µg/ml gave consistently greater responses than other doses tested from 0 to 100 µg/ml. In another experiment not included in this report, inhibition of DNA synthesis was clearly demonstrable with dose levels above 100 µg/ml.

**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Duration of TdR-3H pulse (hr)</th>
<th>Control (cpm/culture)a</th>
<th>IPR (20 µg/ml) (cpm/culture)a</th>
<th>p b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0–48</td>
<td>139,000 ± 5,780</td>
<td>145,000 ± 7,800</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>0–72</td>
<td>210,750 ± 9,620</td>
<td>261,600 ± 17,760</td>
<td>0.005</td>
</tr>
<tr>
<td>2</td>
<td>0–48</td>
<td>44,800 ± 3,050</td>
<td>62,600 ± 2,570</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>0–72</td>
<td>75,200 ± 8,840</td>
<td>106,000 ± 11,670</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>3</td>
<td>20–28</td>
<td>25,800 ± 2,480</td>
<td>35,000 ± 6,606</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>43–51</td>
<td>20,200 ± 2,130</td>
<td>30,600 ± 2,650</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>20–28</td>
<td>18,000 ± 6,160</td>
<td>161,250 ± 60,310</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

aMean of 5 cultures ± S.E.
bStudent's t test.

**DISCUSSION**

Since only the parenchymal cells of the salivary gland respond to IPR, it was important to determine whether these parenchymal cells were present in the cultures. Three properties of salivary glandular epithelium distinguish it from fibroblasts. Epithelial cells in vitro tend to assume a polygonal configuration. In addition, salivary epithelial cells can secrete the specific products sialomucin and amylase. Epithelioid cell foci associated with sialomucin were found in the cultures. Amylase activity was not demonstrated. It is not unusual for specialized cells to lose the ability to make specific products after a brief period in vitro (7). This is a possible explanation for the failure to demonstrate amylase.

In these cultures, the gradual loss of epithelial cells with repeated passages paralleled the gradual loss of response to IPR. It is possible that the epithelial foci were the responsive cells in the cultures, and that their disappearance was the cause of the loss of response. Overgrowth of cultures of epithelial cells by fibroblasts is a well-known phenomenon in tissue culture.

Certainly, the magnitude of the response of early passage cultures to IPR (Table 1), while quite significant, is of modest proportions. This observation is consistent with the interpretation that only small numbers of cells in the cultures are responding to IPR.

In retrospect, an even greater response might have been obtained in the early experiments (Table 1) if the data of the dose-response studies had then been available. The evidence in Chart 1 suggests that the arbitrary dose used in the initial experiments (20 µg/ml) was not as effective as the later demonstrated optimal dose (50 µg/ml).

The data presented in this report indicate that IPR can stimulate the incorporation of TdR-3H into DNA of monolayer cell cultures derived from salivary glands. This IPR-induced stimulation of DNA synthesis is clearly independent of other tissues or organ systems.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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