Phagocytosis of outer segments by cultured rat pigment epithelium

Reduction by cyclic AMP and phosphodiesterase inhibitors

Ross B. Edwards and Suzanne Bakshian

Phagocytosis of isolated outer segments by cultured rat pigment epithelium was decreased by cyclic 3',5'adenosine monophosphate (cyclic AMP), dibutyryl cyclic AMP, and three phosphodiesterase inhibitors (isobutylmethylxanthine, papaverine, and SQ 65442). Cyclic GMP and its dibutyl derivative had little effect on phagocytosis. Phagocytosis was moderately reduced by several other purine nucleotides and nucleosides and substantially reduced by adenine.

Key words: pigment epithelium, phagocytosis, cyclic AMP, cyclic GMP, cell culture, rod outer segments, photoreceptors, rats, phosphodiesterase inhibitors

Cyclic nucleotides affect the phagocytic activity of several kinds of cells. For example, cyclic 3',5'adenosine monophosphate (cyclic AMP) and agents which stimulate its intracellular accumulation reduce the phagocytic activity of polymorphonuclear leukocytes, and macrophages, and macrophage-like cell line P388 D1 and stimulate the phagocytic activity of thyroid follicular cells and macrophage-like cell line J774.7 Cyclic 3',5'-guanosine monophosphate (cyclic GMP) and agents which stimulate its intracellular accumulation stimulate the phagocytic activity of polymorphonuclear leukocytes and macrophages.

One of the functions of the retinal pigment epithelium is to phagocytize the shed tips of photoreceptor outer segments, a process which has been studied in vitro with cultured rat pigment epithelium and isolated rod outer segments. Because of the known effects of cyclic nucleotides on phagocytic activity in other cell types and because cyclic AMP and cyclic GMP concentrations in the pigment epithelium (2 to 3 and 2 to 9 pmol/mg of protein, respectively) are comparable to levels observed in many other tissues, the present study was done to determine the effects of cyclic AMP and cyclic GMP on the phagocytosis of isolated outer segments by cultured rat pigment epithelium. The dibutyl derivatives of these compounds were also tested since they more readily enter cells and are more resistant to degradation by cyclic nucleotide phosphodiesterases than the corresponding underivatized cyclic nucleotides. Inhibitors of phosphodiesterase,
Cyclic AMP, phagocytosis in rat pigment epithelium

which have been shown to increase cyclic nucleotide levels in other tissues,\textsuperscript{18} and compounds structurally related to cyclic AMP and cyclic GMP were also tested.

Methods

Cyclic nucleotides, other purine derivatives, isobutylmethylxanthine (IBMX), and papaverine were obtained from Sigma Chemical Co., St. Louis, Mo. SQ 65442 (1-ethyl-4(ethylthio)-1H pyrazolo[3,4-b]-pyridine-5-carboxylic acid, ethyl ester) was provided by the Squibb Institute for Medical Research, Princeton, N. J. Monolayer cultures of 7-day-old normal (Long-Evans) rat pigment epithelium were prepared as previously described.\textsuperscript{19} Tritium-labeled rat rod outer segments were prepared as described\textsuperscript{12} with the following modifications: 20 µCi of a tritiated amino acid mixture (New England Nuclear Corp.) was injected into each eye, and the rats were sacrificed 4 to 7 days after injection; outer segments were isolated by shaking retinas in culture medium for 1 min, followed by removal of retinal debris by centrifuging the retinal suspension at 150 x g for 1 min; the outer segments in the supernatant were pelleted and washed three times in culture medium (1360 x g for 10 min). The outer segment pellet was resuspended in medium so that 1 ml contained the outer segments from approximately one to two retinas or about 7000 to 33,000 cpm/ml. Outer segments from some preparations were disrupted by freeze-thawing and extensive sonication and combined with cold trichloroacetic acid (final concentration 5%) to ensure that the radioactive amino acids had been incorporated into protein; a minimum of 98% of the radioactivity was precipitable.

For measurement of the phagocytosis of the radioactive outer segments, cultures were preincubated for 30 min with medium containing the compound to be tested; the medium was then replaced with 1 ml of the outer segment suspension containing the compound. Control cultures were treated similarly without added compounds. The pH of all media was 7.4 to 7.7 and was not affected by the added compounds except for 5'-AMP, which required adjustment to 7.4 to 7.7 with 0.1N NaOH. Cultures were incubated with radioactive outer segments for 4 hr, placed on ice, and vigorously rinsed three times with balanced salt solution\textsuperscript{19} containing 0.00125% (v/v) Triton X-100 to remove noningested outer segments. Cultures were homogenized in 0.9% NaCl, sonicated, and assayed for radioactivity by scintillation counting and for protein by the method of Lowry et al.\textsuperscript{21} Results are expressed as counts per minute per

Table I. Effects of phosphodiesterase inhibitors on phagocytosis of radioactive outer segments by normal cultured rat pigment epithelium

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>% Control ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>IBMX, 1 mM</td>
<td>40 ± 6*</td>
</tr>
<tr>
<td>Papaverine, 0.4 mM</td>
<td>51 ± 6*</td>
</tr>
<tr>
<td>Papaverine, 0.1 mM</td>
<td>82 ± 8*</td>
</tr>
<tr>
<td>SQ 65442, 0.4 mM</td>
<td>50 ± 6*</td>
</tr>
</tbody>
</table>

* Significantly different from controls, p < 0.0005. Six cultures were used for each determination.

Fig. 1. Effects of cyclic AMP, cyclic GMP, and their dibutyryl derivatives on phagocytosis of outer segments by cultured normal rat pigment epithelium. The number within each bar is the number of cultures tested for that compound and concentration. Vertical lines equal 1 S.D. Asterisks indicate values which differed from controls with p < 0.01.
milligram of cell protein. Protein content varied between 0.08 and 0.18 mg/culture, and phagocytic values for control cultures ranged from 6000 to 15,000 cpm/mg of protein. The statistical significance of differences between control and experimental values was determined by the one-tailed t test.

To determine the effect of different compounds on the viability of cultured rat pigment epithelium, each compound which had been tested for its effect on phagocytosis was added to the medium of a culture; the cultures were incubated for 4.5 or 24 hr and then immediately incubated for 10 min at 37°C with 0.04% trypan blue dissolved in balanced salt solution. Cells which did not take up the dye were considered viable.21

Results

Cyclic AMP reduced the phagocytosis of isolated rod outer segments by cultured rat pigment epithelium; at 1 mM, 5 mM, and 25 mM, amounts phagocytized were 73%, 67%, and 51%, respectively, of control values (Fig. 1). Dibutyryl cyclic AMP at the same concentrations was more effective than cyclic AMP; 1 mM reduced phagocytosis to 55% of control values (Fig. 1). Cyclic GMP, in contrast, had a much smaller effect than cyclic AMP; at 5 mM the amount phagocytized was not significantly different from that of control cultures. Likewise, 1 mM dibutryl cyclic GMP did not significantly reduce phagocytosis and at 5 mM and 25 mM reduced phagocytosis less than did the same concentrations of cyclic AMP or dibutryl cyclic AMP (Fig. 1).

Inhibitors of cyclic nucleotide phosphodiesterase were also effective in reducing phagocytosis. A decrease in phagocytosis to 50% or less of control values was observed with IBMX, papaverine, and SQ 65442 (Table 1).

The effects on phagocytosis of other compounds structurally related to cyclic AMP and cyclic GMP were also tested. The decrease due to 5'-AMP (5 mM) was the same as by cyclic AMP (5 mM) (Fig. 1). Adenosine and 3'-AMP (also 5 mM) reduced phagocytosis slightly less than did cyclic AMP; the same concentration of guanosine and 5'-GMP also resulted in statistically significant decreases compared to both control values and 5 mM cyclic GMP (Fig. 2). Adenine (5 mM) had a marked effect, reducing phagocytosis to 30% ± 5 of control values (6 cultures, p < 0.0005).

None of the compounds resulted in detectable cell death at the concentrations tested as measured by the trypan blue exclusion test, at either 4.5 or 24 hr of exposure to these compounds.

Discussion

The present study shows that cyclic AMP reduced the phagocytosis of isolated rat rod outer segments by cultured rat pigment epithelium, whereas cyclic GMP did not affect phagocytosis. In addition, the phosphodiesterase inhibitors IBMX,22 papaverine,18 and SQ 65442 (R. N. Lolley, personal communication), known to increase cyclic AMP and cyclic GMP in other cell types, also reduced
phagocytosis in vitro, further supporting the idea that phagocytosis can be reduced by increasing the intracellular concentration of cyclic nucleotides.

Although cyclic GMP did not affect phagocytosis, its dibutyryl derivative did reduce phagocytosis, but to a lesser extent than cyclic AMP or dibutyl cyclic AMP. The differences between the effects of the cyclic nucleotides and their respective dibutyryl derivatives could be because the latter more readily enter cells and better resist degradation by phosphodiesterases than the undervatized cyclic nucleotides. Since the intracellular concentrations of the cyclic nucleotides were not measured, it is not yet established whether these effects in vitro were achieved with concentrations comparable to those known to exist in vivo.

The effectiveness of adenosine and guanosine derivatives in reducing phagocytosis did not appear to depend on the presence of the phosphodiester ring. Specifically, the reductions in phagocytosis seen with adenosine, 5'AMP, 3'-AMP, guanosine, and 5'-GMP at 5 mM were comparable to the decrease due to 5 mM cyclic AMP. In addition, adenine also reduced phagocytosis. In the case of adenosine, 5'-AMP, and 3'-AMP, it is possible that the reduction of phagocytosis was mediated through the stimulation of cyclic AMP synthesis, as these compounds have been shown to stimulate cyclic AMP synthesis in brain slices or in isolated epidermis. Experiments are in progress to determine whether intracellular cyclic AMP is increased by these compounds in cultured rat pigment epithelium.

We thank the Squibb Institute for Medical Research, Princeton, N. J., for providing the phosphodiesterase inhibitor SQ 65442.

REFERENCES


20. Lowry OH, Rosebrough NJ, Farr AL, and Randall

