Prostaglandins (PG) of the E series and catecholamines stimulate adenosine 3'-5'-monophosphate (cAMP) formation in human astrocytoma cells (1321N1). These two classes of effectors activated adenylate cyclase upon interaction with different receptor systems. No evidence for a mediatory role for PG in the action of catecholamines was found. PG interacted with 1321N1 cells with an order of potency of PGE \textsubscript{1} = PGE \textsubscript{2} > PGA \textsubscript{1} > PGF \textsubscript{2a}. The effect of combinations of the various PG indicated that all efficacious PG interacted with a common receptor. 7-Oxa-13-prostenoic acid and indomethacin were shown to be competitive inhibitors of the effect of PGE \textsubscript{1}, with \( K \textsubscript{i} \) values of 4 and 150 \( \mu \text{M} \), respectively. These two compounds did not inhibit the effect of isoproterenol. Polyphloretin phosphate caused a complex pattern of inhibition of the effects of PGE, and at higher concentrations also inhibited the effects of isoproterenol. The mefenamic acid class of nonsteroidal anti-inflammatory agents was found to inhibit the effects of PGE, with a potency order of meclofenamic acid > flufenamic acid = mefenamic acid. The inhibitory action of meclofenamic acid was complex involving specific, but partial, insurmountable antagonism of PGE, as well as competitive inhibition of PGE, effects. At higher concentrations of meclofenamic acid a nonspecific inhibition of the effects of both PGE \textsubscript{1} and isoproterenol was observed. These two classes did not inhibit the effect of isoproterenol. The mefenamic acid class of nonsteroidal anti-inflammatory agents was found to inhibit the effects of PGE, with a potency order of meclofenamic acid > flufenamic acid = mefenamic acid. The inhibitory action of meclofenamic acid was complex involving specific, but partial, insurmountable antagonism of PGE, as well as competitive inhibition of PGE, effects. At higher concentrations of meclofenamic acid a nonspecific inhibition of the effects of both PGE \textsubscript{1} and isoproterenol was observed. These studies suggest that the inhibition by nonsteroidal anti-inflammatory agents of the physiological effects of PGE, in animals may occur, at least in part, at the level of adenylate cyclase. The possibility that multiple classes of adenylate cyclase-linked PGE receptors might exist in nature is discussed.\[\text{\( \rightleftharpoons \)}\]

Prostaglandins (PG) have been shown to modify the intracellular concentration of adenosine 3'-5'-monophosphate (cAMP) in a variety of tissues and cultured cell lines (1, 2). Prostaglandins of the E series, PGE \textsubscript{1} and PGE \textsubscript{2}, can increase cAMP levels, presumably by direct stimulation of adenylate cyclase activity (3, 4), or can decrease cAMP levels by a mechanism that is not understood (4). In at least two tissues (5, 6) results have been obtained suggesting that prostaglandins are obligate intermediates in the actions of certain polypeptide hormones as stimulators of adenylate cyclase.

The direct binding of radioactively labeled prostaglandins to broken cell preparations of a variety of tissues (7-14) and to intact cells (15) has been reported. However, in most of these studies the relation of the prostaglandin "receptor" and adenylate cyclase could not be assessed; primarily because the relative effects on adenylate cyclase of the various prostaglandin analogues were not determined. Recently, Brunton et al. (16) have demonstrated a convincing correlation between the binding of prostaglandins of the E series and the activation of adenylate cyclase in three cultured murine cell lines. Thus, it would appear that activation of adenylate cyclase upon interaction with structure specific receptors is the basis of at least a part of the physiological effects of PGE \textsubscript{1} and PGE \textsubscript{2}.

Nonsteroidal anti-inflammatory agents have been shown to inhibit prostaglandin biosynthesis in a number of tissue systems (17). Although this effect is presumed to be the basis for the mechanism of action of this class of compounds recent reports have shown that nonsteroidal anti-inflammatory agents also inhibit the actions of prostaglandins. Levy and Lindner (18) found that meclofenamic acid selectively blocked the systemic vasodepressor effects of PGF \textsubscript{2a}, in the rabbit but had no effect on the response to PGE \textsubscript{1}. Koss et al. (19) also observed rather selective blockade by meclofenamic acid of PGF \textsubscript{2a}, induced reflex bradycardia and hypotension in cats. On the other hand, others (20-22) have observed that nonsteroidal anti-inflammatory agents inhibit PGE \textsubscript{1} and PGE \textsubscript{2}-induced contractions of guinea pig ileum and uterus and gerbil colon preparations. Toiman and Partridge (22) also observed that indomethacin and mefenamic acid competed with PGE for binding to broken cell preparations of rat epididymal adipocytes. However, a detailed analysis of the effects of nonsteroidal anti-inflammatory agents on prostaglandin-stimulated adenylate cyclase activity has not been reported to date.

In the present study we have examined the effects of certain

\[\text{\( \rightleftharpoons \)}\]
concluded that a single type of receptor mediates the actions of tynoic acid, polyphloretin phosphate, indomethacin, and a nonsteroidal anti-inflammatory agents as well as other compounds on the action of prostaglandins to stimulate cAMP formation in human astrocytoma cells in culture. The pharmacological characteristics of the receptor mediating the actions of the prostaglandins are established and the kinetics and structural specificity of the inhibitory actions of 7-oxa-13-prostaglandin A, and 7-oxa-13-prostaglandin F, were kindly provided by Ayerst Research Laboratory, Montreal, and 7-oxa-13-prostaglandin A, and 7-oxa-13-prostaglandin F, were kindly provided by Dr. J. Fried, University of Chicago. SC 19920 was a gift from Searle Corp., Chicago, III., polyphloretin phosphate was obtained from A. B. Loo, Helsingborg, Sweden. The nonsteroidal anti-inflammatory agents phenylbutazone, oxyphenbutazone, and Voltaren were donated by CIBA-GEIGY, Summit, N. J.; meclofenamic, flufenamic, and mefenamic acids were obtained from Parke Davis, Detroit, Mich.; indomethacin from Merck, Sharp and Dohme, West Point, Pa. Aspirin, arachidonic acid, and (±)-isoproterenol were purchased from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co. and [14C]adenine (52 mCi/mmol) was purchased from Schwarz/Mann.

**Materials** — Prostaglandins E, F, A, and F were a gift from Dr. J. Pike, Upjohn Co., Kalamazoo, Mich.; C-15 stereoisomers of 10-11, 13-14-tetrahydro-PGA, and 10-11-dihydro-PGA, were kindly provided by Ayerst Research Laboratory, Montreal, and 7-oxa-13-prostaglandin A, and 7-oxa-13-prostaglandin F, was provided by Dr. J. Fried, University of Chicago. SC 19920 was a gift from Searle Corp., Chicago, Ill., polyphloretin phosphate was obtained from A. B. Leo, Helsingborg, Sweden. The nonsteroidal anti-inflammatory agents phenylbutazone, oxyphenbutazone, and Voltaren were donated by CIBA-GEIGY, Summit, N. J.; meclofenamic, flufenamic, and mefenamic acids were obtained from Parke Davis, Detroit, Mich.; indomethacin from Merck, Sharp and Dohme, West Point, Pa. Aspirin, arachidonic acid, and (±)-isoproterenol were purchased from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co. and [14C]adenine (52 mCi/mmol) was purchased from Schwarz/Mann.

**Cell Culture Conditions** — The origin and growth conditions of the 1321N1 human astrocytoma cell line have been described (23, 24). For experiments, cells were grown in 60- or 35-mm Falcon plastic culture dishes. Dishes (60 mm) received 5 ml of Dulbecco's modified Eagle's minimal essential medium supplemented with 5% fetal calf serum in an atmosphere of 93% air and 7% CO, at 37° in a humidified incubator; 35-mm dishes received 2 ml of medium. Culture dishes (60 mm) were seeded with 3 to 6 x 10^5 cells per dish and grown for 4 to 6 days without changing the medium. Cell numbers were determined with a model A Coulter counter.

**Experimental Incubation Conditions** — Cells in 35-mm culture dishes received 0.1 μCi of [14C]adenine in 1.0 ml of serum-free growth medium; cells in 60-mm dishes received 1.0 μCi in 5 ml of serum-free medium. After 60 min at 37° the medium was aspirated, the cells washed with serum-free growth medium to remove excess [14C]adenine, and exposed for 5 min at 37° in 1 ml (35-mm dishes) or 5 ml (60-mm dishes) growth medium without serum to the indicated concentrations of test agents. Test agents were dissolved in 0.01 N HCl or 95% ethanol and diluted 100-fold into the incubation medium. At the concentration used (1%) none of the solvents had a detectable effect.

**TABLE I**

<table>
<thead>
<tr>
<th>Prostaglandins</th>
<th>Maximal response</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>% PGE&lt;sub&gt;1&lt;/sub&gt;</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>100</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>49</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE&lt;sub&gt;3&lt;/sub&gt;</td>
<td>31</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-11, 13-14-Tetrahydro-PGA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>39</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-Epi, 10-11, 13-14-tetrahydro-PGA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>9</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-11-Dihydro-PGA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>30</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-Epi, 10-11-dihydro-PGA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE&lt;sub&gt;5&lt;/sub&gt;</td>
<td>No detectable change at 100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Not determined.

**EXPERIMENTAL PROCEDURES**

**Materials** — Prostaglandins E, F, A, and F were a gift from Dr. J. Pike, Upjohn Co., Kalamazoo, Mich.; C-15 stereoisomers of 10-11, 13-14-tetrahydro-PGA, and 10-11-dihydro-PGA, were kindly provided by Ayerst Research Laboratory, Montreal, and 7-oxa-13-prostaglandin A, and 7-oxa-13-prostaglandin F, was provided by Dr. J. Fried, University of Chicago. SC 19920 was a gift from Searle Corp., Chicago, Ill., polyphloretin phosphate was obtained from A. B. Leo, Helsingborg, Sweden. The nonsteroidal anti-inflammatory agents phenylbutazone, oxyphenbutazone, and Voltaren were donated by CIBA-GEIGY, Summit, N. J.; meclofenamic, flufenamic, and mefenamic acids were obtained from Parke Davis, Detroit, Mich.; indomethacin from Merck, Sharp and Dohme, West Point, Pa. Aspirin, arachidonic acid, and (±)-isoproterenol were purchased from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co. and [14C]adenine (52 mCi/mmol) was purchased from Schwarz/Mann.

**Experimental Incubation Conditions** — Cells in 35-mm culture dishes received 0.1 μCi of [14C]adenine in 1.0 ml of serum-free growth medium; cells in 60-mm dishes received 1.0 μCi in 5 ml of serum-free medium. After 60 min at 37° the medium was aspirated, the cells washed with serum-free growth medium to remove excess [14C]adenine, and exposed for 5 min at 37° in 1 ml (35-mm dishes) or 5 ml (60-mm dishes) growth medium without serum to the indicated concentrations of test agents. Test agents were dissolved in 0.01 N HCl or 95% ethanol and diluted 100-fold into the incubation medium. At the concentration used (1%) none of the solvents had a detectable effect.

**Fig. 1.** Time course of intracellular and extracellular cAMP accumulation in cultures of 1321N1 cells exposed to 100 μM PGE<sub>1</sub>. The per cent conversion of intracellular [14C]ATP to [14C]cAMP (●) and the per cent of total [14C]cAMP in the medium are shown (○). Values are the mean of duplicate determinations. Vertical bars indicate the range of duplicates. Inset, the effect of a 10-min exposure to increasing PGE<sub>1</sub> concentrations on the cAMP content of 1321N1 cells. As determined by the method of Gilman basal levels of cAMP under these experimental conditions ranged from 10 to 15 pmol/mg of protein. PGE<sub>1</sub> stimulated maximal accumulations of 500 to 700 pmol/mg of protein.

**Fig. 2.** Effect of PGA<sub>1</sub> on the cAMP content of 1321N1 cells in the absence and presence of PGE<sub>1</sub>. A, the effect of PGA<sub>1</sub> alone (●), PGE<sub>1</sub> in the presence of 1.0 μM PGE<sub>1</sub> (●), and the theoretical curve for additivity of the effects of PGA<sub>1</sub> and PGE<sub>1</sub> (○) are shown. The effect of 1.0 μM PGE<sub>1</sub>, alone was 1.5% conversion. Values are the mean of duplicate determinations. B, theoretical curves for the stimulation of cAMP accumulation in 1321N1 cells by PGA<sub>1</sub> in the absence (●) and presence (○) of PGE<sub>1</sub> are shown. The values were calculated under the assumption that PGA<sub>1</sub> (EC<sub>50</sub> = 9 μM, V<sub>max</sub> = 1.5% conversion) and PGE<sub>1</sub> (EC<sub>50</sub> = 2 μM, V<sub>max</sub> = 5.0% conversion) act competitively at the same receptor site. For the calculation see Footnote 1.
Table II

<table>
<thead>
<tr>
<th>PGE1</th>
<th>Agonist (100 µM)</th>
<th>Per cent conversion</th>
<th>Calculated for additivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found</td>
<td>2.83 ± 0.07a</td>
</tr>
<tr>
<td>1.0</td>
<td>10-11, 13-14-Tetrahydro-PGA1</td>
<td>3.41 ± 0.02</td>
<td>2.40 ± 0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>15-Epi, 10-11, 13-14-tetrahydro-PGA1</td>
<td>2.64 ± 0.08</td>
<td>2.83</td>
</tr>
<tr>
<td>1.0</td>
<td>Isoproterenol</td>
<td>2.00 ± 0.10</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>1.0</td>
<td>Isoproterenol</td>
<td>1.56 ± 0.04</td>
<td>2.64 ± 0.01</td>
</tr>
<tr>
<td>100</td>
<td>Isoproterenol</td>
<td>3.86 ± 0.13</td>
<td>4.15 ± 0.01</td>
</tr>
</tbody>
</table>

* Mean ± S.E. (n = 3).

Table III

<table>
<thead>
<tr>
<th>Agonist</th>
<th>7-Oxa-13-prostynoic acid</th>
<th>Per cent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE1</td>
<td>100</td>
<td>26 ± 4*</td>
</tr>
<tr>
<td>PGE1</td>
<td>100</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>10-11, 13-14-Tetrahydro-PGA,</td>
<td>100</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>10-11-Dihydro-PGA,</td>
<td>100</td>
<td>22 ± 0.2</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>100</td>
<td>114 ± 6</td>
</tr>
</tbody>
</table>

* Mean ± S.E. (n = 3).

influence on the CAMP content of 1321N1 cells. Unless otherwise stated, agonists and antagonists were added simultaneously.

Measurement of Total CAMP and [3H]CAMP—Experimental incubations were stopped by aspirating the medium and adding 1.0 ml of 6% trichloroacetic acid. The acid extract was applied to a column (0.4 cm × 4 cm) of Dowex (Bio-Rad Ag 50W-X8-H form, washed with 1.0 N HCl and water). ATP and ADP were eluted by addition of 1.5 ml of 0.1 N HCl and 1.5 ml of HClO4; CAMP was eluted with an additional 3 ml of water. After addition of 0.1 ml of 1.5 M Tris/HCl buffer, pH 7.8, the CAMP-containing fraction was further purified on columns (0.4 cm × 3 cm) of neutral alumina (25). Columns were washed with 1.0 ml of 0.05 M Tris/HCl, pH 7.8, and the combined eluates counted in 8 ml of dioxane containing 6 g of 2,5-diphenyloxazole (PPO), 60 g of naphthalene/liter of dioxane, by liquid scintillation spectrometry. The counting efficiency was 78%. Overall recovery for CAMP was 70 ± 8%. Results are reported as "% conversion." Such values represent (dpm of [3H]CAMP × 100)/dpm of [3H]CAMP + dpm of [3H]ATP.

The validity of the prelabeling assay was confirmed for each different experimental design by determination of the total CAMP content of purified samples using the Gilman (26) binding assay. Data from prelabeling assays are presented since the variability in replicate determinations was found to be significantly less than with the binding assay. A detailed description of the labeling conditions and the kinetic characteristics of the incorporation of label into ATP and cAMP has been presented elsewhere (24, 27).

Determination of Effects of PGE, on Suspended Cells—Cells were incubated with [3H]Clodamine as described above then incubated with 0.25% trypsin in sodium citrate buffer, pH 6.8, for 30 s. The trypsin solution was then aspirated and the culture incubated at 37° for 5 min. The cells were suspended in growth medium and washed twice by centrifugation and resuspension in growth medium. The cells were finally suspended in serum-free growth medium at a density of 1 to 5 × 106 cells/ml. Experimental incubations were started by addition of PGE, to the cell suspension in a rapidly shaking water-bath at 37° with a 95% air, 5% CO2 atmosphere. At the indicated time

200-μl aliquots were removed and pipetted into 0.8 ml of 6.25% trichloroacetic acid. The supernatant was analyzed for [3H]CAMP and [3H]ATP as described above.

Suspended cells were used to measure the kinetics of the onset of CAMP production upon addition of PGE,; the kinetics of the onset of inhibition by 7-oxa-13-prostynoic acid and meclofenamic acid, and the reversal of such inhibition by excess PGE, (Fig. 4). In preliminary experiments the properties of the suspended cells were studied in detail to determine whether the response of attached and suspended cells were similar. The results of these studies can be summarized as follows:

1. The maximal amount of CAMP produced by suspended cells was about 86% of the CAMP produced by attached cells (700 pmol of CAMP/mg of protein/5 min).
2. The concentrations of PGE1, PGE2, and isoproterenol that effected a half-maximal response (EC50) were the same for both attached and suspended cells.
3. The specific activity of [3H]CAMP accumulated after exposure to PGE1 was the same in attached and suspended cells.
4. The time course of CAMP accumulation after exposure to PGE1 was similar and exhibited the same biphasic pattern.
5. The ratio of intra- to extracellular [3H]CAMP after exposure to PGE1 was the same.
6. The response of suspended cells to PGE1 did not change during the first 90 min after suspension of cells. The [3H]ATP content of suspended cells declined only by about 15% during a 90-min incubation.

RESULTS

Previous reports from our laboratory have shown that human astrocytoma cells (1321N1) respond to catecholamines (24, 25, 29), and adenosine (29) with a marked accumulation of CAMP. The cells also respond to prostaglandins as shown in Fig. 1 which illustrates the time course of intra- and extracellular accumulation of CAMP in the presence of maximally effective concentrations of PGE1. The inset illustrates the concentration-effect relationships when 1321N1 cells are incubated for 10 min with different concentrations of PGE1. The decline in cellular CAMP that occurs even in the continued presence of PGE1 (Fig. 1) has been shown to be due to PGE1-induced desensitization of adenylate cyclase (25, 28).

Unless otherwise stated, the "response" to an agonist was determined by measuring the intracellular CAMP after a 10-min incubation. The excrcion of CAMP into the medium during the first 10 min of exposure to PGE1 did not exceed 1% of the total CAMP present in the incubation at that time.

Structure-Function Relationships—The effects of different prostaglandins on the accumulation of CAMP are summarized in Table I. PGE1 caused the greatest increase in CAMP content. Introduction of the C14 double bond (PGD2) reduced the maximal effect but did not change the apparent affinity. Removal of the hydroxy group at C9, and introduction of a C9-C11 double bond (PGI2) reduced both the maximal effect and the apparent affinity, while the reduction of the C9 keto group (PGE2) abolished all agonist activity. The presence or absence
Regulation of Adenylate Cyclase by Prostaglandins

Fig. 3. Inhibition by 7-oxa-13-prostynoic acid of the effects of PGE\textsubscript{1} on cAMP accumulation in 1321N1 cells. A, concentration-effect curves in the absence (●) and in the presence of 20 μM (●), 50 μM (□), and 100 μM (△) 7-oxa-13-prostynoic acid are shown. Each value is the mean of duplicate determinations. The vertical bars represent the range of duplicates. Inset, Schild plot of values taken from the curves at a response level of 3.0%. B, Lineweaver-Burk analysis of the inhibition of the effect of PGE\textsubscript{1} by 7-oxa-13-prostynoic acid. The response of the cells to PGE\textsubscript{1}, in the absence (●) and in the presence of 20 μM (□), 50 μM (△), and 75 μM (○) 7-oxa-13-prostynoic acid is shown. Each value is the mean of duplicate or triplicate determinations. The lines have been fitted by least square regression analysis.

Fig. 4. Time course of the onset and the reversal of the inhibition of PGE\textsubscript{1} stimulated accumulation of cAMP by 7-oxa-13-prostynoic acid and meclofenamic acid in suspended 1321N1 cells. Cells (4.6 × 10\textsuperscript{6}) were suspended by trypsinization in 6.2 ml of serum-free growth medium as described under "Experimental Procedures." The cell suspension was stirred at 37° and 200-μl aliquots removed at the indicated times after the addition of 1.0 μM PGE\textsubscript{1}, 7-oxa-13-prostynoic acid (50 μM) was added after 45 s (arrow) and 300 μM PGE\textsubscript{1} after 125 s (arrow); B, meclofenamic acid (52 μM) was added after 45 s (arrow) and 300 μM PGE\textsubscript{1} after 125 s (arrow). Values represent single determinations.

of the C\textsubscript{10-11} double bond did not appear to influence the effect of prostaglandins on 1321N1 cells. Experiments with 15-epi prostaglandins suggest that the receptor site is selective for compounds with the α configuration at C\textsubscript{10}.

Analysis of Interaction of Prostaglandin Agonists—Previous studies by Kantor et al. (30) suggested the presence, in rabbit intestinal epithelial cells, of two independent receptors for prostaglandins; one that mediated activation and another that mediated inhibition of adenylate cyclase activity. In their studies PGE\textsubscript{1} was selective for the activating receptor while PGA\textsubscript{1} was selectively inhibitory. The prostaglandin analogue 7-oxa-13-prostynoic acid was stimulatory at low concentrations and inhibitory at high concentrations. No evidence for such a circumstance was observed in the present studies with 1321N1 cells. Fig. 2 illustrates an experiment in which cells were incubated with increasing amounts of PGA\textsubscript{1} in the presence or absence of 1.0 μM PGE\textsubscript{1}. The results can be compared with the effect expected if both compounds interact as agonists with independent receptors (Fig. 2A, dashed line) or with the same receptor (Fig. 2B). All experiments involving co-addition of...
Reversibility of inhibitory action of prostaglandin inhibitors by washing

The cells were preincubated for 15 min with the indicated concentrations of inhibitors; control cells were incubated with solvent (ethanol). After aspirating the medium all cultures including controls were washed four times with 2 ml of serum-free growth medium and then exposed to the indicated concentrations of PGE, for 5 min. Control dishes received only PGE,. Cultures exposed to PGE, and inhibitors simultaneously were not preincubated with inhibitors. In unwashed cultures incubated with the indicated concentrations of inhibitors the degree of inhibition was polyphloretin phosphate 98%, 7-oxa-13-prostynoic acid 89%, meclofenamic acid 98%.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PGE,</th>
<th>Inhibitor + PGE, added simultaneously</th>
<th>Cells preincubated 15 min with inhibitors then washed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphloretin phosphate (300 ng/ml)</td>
<td>1</td>
<td>18 ± 1</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>7-Oxa-13-prostynoic acid (100 µM)</td>
<td>10</td>
<td>38 ± 2</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>Meclofenamic acid (100 µM)</td>
<td>1</td>
<td>8 ± 1</td>
<td>91 ± 2</td>
</tr>
</tbody>
</table>

*Mean ± S.E. (n = 3).

Different prostaglandins were conducted under conditions where the capacity of the cells to respond was not exceeded, i.e. the effect of both agonists added together was less than the maximal effect of PGE, (100 µM). The effects of two agonists acting through the same receptor should be less than additive under these conditions, whereas agonists acting at independent receptor sites should produce additive effects (31). The results (Fig. 2) indicate that PGE, and PGA, acted as agonists at all concentrations tested and apparently interacted with a common receptor. PGA, can act as a partial agonist of PGE, under appropriate conditions (not illustrated) because its apparent affinity for the receptor is similar (EC50 9 µM) to that of PGE, (EC50 2 µM) but it has only 30% of the intrinsic activity of PGE,.

The results of experiments summarized in Table II indicate that all prostaglandins that stimulated CAMP accumulation in 1321N1 cells produced less than additive effects together with 1.0 µM PGE, or PGA, but not act as an agonist nor did it act as an antagonist of PGE, even at a 100-fold excess concentration. These results (Fig. 2, Table II) suggest that only one type of receptor mediates the effects of prostaglandins on cAMP formation in 1321N1 cells.

Results summarized in Table II indicate that the effect of 100 µM isoproterenol was additive to the effect of a low concentration (1.0 µM) of PGE, but was less than additive when present with 100 µM PGE, or PGE, or PGA, (not illustrated). Thus, the effects of catecholamines and prostaglandins were additive except at high concentrations of both agonists, where the capacity of adenylate cyclase to be stimulated might be exceeded.

Concentrations of propranolol that completely blocked the effects of catecholamines on CAMP content in 1321N1 cells had no effect on the response of the cells to PGE, or PGE, (not illustrated). Also, 7-oxa-13-prostynoic acid, a potent competitive inhibitor of PGE, action, had no significant inhibitory effect on the agonist activity of isoproterenol (Table III). Thus, the effects of catecholamines and prostaglandins would appear to be mediated by independent receptors. No evidence was obtained to suggest that prostaglandins are obligate intermediates in the action of catecholamines to stimulate adenylate cyclase activity in 1321N1 cells.

**Analysis of Effects of Antagonists of Prostaglandin Action**—7-Oxa-13-prostynoic acid has been shown to be an inhibi-
Regulation of Adenylate Cyclase by Prostaglandins

The inhibition by this analogue of the accumulation of cAMP, as summarized in Table III, is nearly complete (more than 90%) at a concentration of 500 μM. The inhibition by 7-oxa-13-prostynoic acid of the effects of various prostaglandins is also indicated in Fig. 9. Complete reversal of the inhibitory effect of 100 μM 7-oxa-13-prostynoic acid was observed after adequate washing of attached cells.

**TABLE V**

Effects of nonsteroidal anti-inflammatory agents on cAMP accumulation in 1321N1 cells stimulated by PGE1 or isoproterenol

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC50 PGE1 (μM)</th>
<th>IC50 Isoproterenol (μM)</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mefenamic acid</td>
<td>30 ± 2</td>
<td>86 ± 4</td>
<td>15</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>30 ± 3</td>
<td>92 ± 2</td>
<td>10</td>
</tr>
<tr>
<td>Voltaren</td>
<td>30 ± 7</td>
<td>98 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>100 ± 1</td>
<td>100 ± 10</td>
<td>100</td>
</tr>
<tr>
<td>Phenybutazone</td>
<td>300 ± 8</td>
<td>94 ± 6</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Oxyphenbutazone</td>
<td>300 ± 8</td>
<td>92 ± 7</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Aspirin</td>
<td>1000 ± 5</td>
<td>98 ± 2</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

* Mean ± S.E. (n = 3).

**Fig. 6.** Lineweaver-Burk analysis of the inhibition of the effect of PGE1 by indomethacin. The response of cells to PGE1 in the absence (○) and in the presence of 500 μM (△), 300 μM (★), and 100 μM (■) indomethacin is shown. Each value is the mean of duplicate determinations. The lines have been fitted by least square regression analysis.

**Fig. 7.** Effects of polyphloretin phosphate on cAMP accumulation in 1321N1 cells stimulated by PGE1 (A) and isoproterenol (B). Concentration-effect curves in the absence (△) and in the presence of 50 μg/ml (○), 100 μg/ml (■), and 300 μg/ml (★) of polyphloretin phosphate are shown. Each value represents the mean of duplicate determinations. The vertical bars represent the range of duplicates.
isoproterenol are summarized in Table V. All of the agents tested exhibited preferential inhibition of the effects of PGE,
with the exception of the butazones, and aspirin, which was not inhibitory up to a concentration of 1000 μm.

Polyphloretin phosphate, a mixture of phosphorylated polymers of the dihydrochalcone, phlorizin, has been shown to inhibit a variety of the actions of prostaglandins (17). In Fig. 7 is illustrated the effect of polyphloretin phosphate on the concentration-effect relationships for PGE, (Fig. 7A) and isoproterenol (Fig. 7B). This agent also exhibited a complicated pattern of inhibition of the effects of PGE, similar to the actions of 7-oxa-13-prostynoic acid (compare Fig. 5A). Whereas polyphloretin phosphate was a much more potent inhibitor of the effects of PGE,, it did cause a depression of the maximal response of 1321N1 cells to isoproterenol (Fig. 7B). The effects of this agent were not readily reversed by washing of attached cells (Table IV).

DISCUSSION

The stimulatory effect of prostaglandins on adenylate cyclase provides the simplest experimental system for studies of at least one aspect of the mechanism of action of this important class of compounds. In this regard, the human astrocytoma cell line, 1321N1, would appear to be a suitable system for the pharmacological characterization of the "receptor" mediating the interaction of prostaglandin agonists and antagonists with adenylate cyclase. The cells are a clonal line and, therefore, represent a homogenous population of cells. This should present an advantage in comparison with the use of tissue preparations for similar studies. Also, the adenylate cyclase of 1321N1 cells is stimulated not only by prostaglandins but by catecholamines and adenosine, allowing an analysis of the specificity of putative prostaglandin antagonists.

The response of intact 1321N1 cells to prostaglandins is similar to the response of the cells to catecholamines. Thus, the change in cAMP content is biphasic with an initial rapid rise followed by a slow decline back toward basal levels even in the continued presence of active prostaglandin. This "desensitization" phenomenon has been studied in detail (25, 28) and the mechanisms of desensitization to prostaglandins and to catecholamines appear to be similar. In spite of the similarity of the cellular response to prostaglandins and catecholamines, the effects of the two agonists are mediated by receptors with distinctly different pharmacological characteristics. This conclusion is based on the observations that highly specific antagonists exist for each class of agonist and that the effects of mixtures of PGE and isoproterenol are strictly additive under appropriate conditions. It is consistent with our observations and those of others to suggest that prostaglandins stimulate adenylate cyclase as a result of the interaction with structure-specific receptors that are linked to the enzyme. Recently, Brunton et al. (16) have clearly demonstrated a correlation between the binding of prostaglandins to particulate receptors and their capacity to activate adenylate cyclase. However, the prostaglandin receptor studied by Brunton et al. (16) appears to have a different structural specificity for prostaglandins than the receptor of 1321N1 cells (see below).

The interesting observation of the existence of both stimulatory and inhibitory receptors for prostaglandins in a single cell (30) was not confirmed in our studies. All prostaglandins with agonistic activity tested appeared to interact with a single class of receptors; no evidence for an inhibitory receptor was obtained in these studies.

Kuehl et al. (5) have suggested that activation of an ovarian prostaglandin receptor site is an essential requirement in the action of luteinizing hormone to stimulate cAMP formation. The studies of Sato et al. (6) suggest that a similar relationship exists between thyroid-stimulating hormone and prostaglandins in the thyroid. In both studies the effects of the hormones and the prostaglandins on cAMP formation were found not to be additive and inhibitors of the effects of prostaglandins, e.g., 7-oxa-13-prostynoic acid, were observed to block the effects of the hormones. We have conducted similar experiments and find no evidence for an intermediary role for prostaglandins in the stimulatory actions of catecholamines on adenylate cyclase activity in 1321N1 cells.

The literature related to prostaglandin antagonists has been reviewed recently (33). Many of these studies have involved an analysis of the effects of antagonists on the physiological response of various smooth muscle preparations to prostaglandins. The results of such studies are difficult to assess in terms of the mechanism of antagonism since the inhibitors might not have interacted exclusively with the putative prostaglandin receptor, but might have influenced, as well, intermediate steps between binding at the receptor and the ultimate effect that was measured, e.g., muscle contraction. Since the synthesis of cAMP is the first measurable event resulting from the interaction of prostaglandins with the adenylate cyclase system, it seemed appropriate to examine the effects of putative prostaglandin antagonists in terms of their inhibition of cAMP synthesis.

Based on our kinetic analysis, the prostaglandin analogue 7-oxa-13-prostynoic acid appeared to be a specific competitive inhibitor of the effects of prostaglandins on cAMP formation. The rate of onset of its action was rapid and the inhibitory state was completely and rapidly reversible. Over the range of concentrations tested (1 to 300 μm) 7-oxa-13-prostynoic acid did not stimulate the formation of cAMP. In this study, 7-oxa-13-prostynoic acid was significantly more potent (Kᵢ = 4 μm) as an antagonist of the effects of PGE₂ on 1321N1 cells than it was found to be in competition with [³H]PGE₂, for binding (Kᵢ about 100 μm) in other cultured cell lines (16). The significance of this discrepancy is not clear at this time (however, see below). Our observations are consistent with other studies that have shown 7 oxa 13 prostynoic acid to be a potent inhibitor of the effects of prostaglandins on cAMP formation in mouse ovaries, pituitary, thyroid slices, toad bladder, and monkey granulosa cell cultures (33).

Polyphloretin phosphate also has been shown to be an inhibitor of the effects of prostaglandins in a variety of experimental systems (17). Eakins (34) showed that this agent acts as a specific, reversible, surmountable antagonist of the effects of both PGE₂ and PGE₃, in jird colon. It was later shown that the low molecular weight fraction of polyphloretin phosphate contained the active antagonist (35). Preliminary results from our studies indicate that 4-dihlorophenyl phosphate is a more potent inhibitor of the effects of PGE₂ on 1321N1 cells than is polyphloretin phosphate. At a concentration of 6.0 μm the dimer inhibited by 50% the effect of 1.0 μm PGE₂. It caused 50% inhibition of the effect of 0.1 μm isoproterenol at a concentration of 113 μm. On the other hand the monomer, 4-phlorophenyl phosphate, was about 100 times less potent as an inhibitor of the effect of PGE₂. These results suggest that the low molecular weight polymers of phloroquin may be the most active components of polyphloretin phosphate.

In the present studies polyphloretin phosphate appeared to inhibit the effects of PGE₂ by more than one mechanism. At lower concentrations it was highly specific as a PGE₂ antagonistic activity tested appeared to interact with a single cell (30) was not confirmed in our studies. All prostaglandins with agonistic activity tested appeared to interact with a single class of receptors; no evidence for an inhibitory receptor was obtained in these studies.
nast and its effects could be interpreted to result from competitive inhibition. However, at higher concentrations polyphlorotetrahydroquinone phosphate was a rather nonspecific, insurmountable antagonist of the effects of both PGE\textsubscript{1} and isoproterenol. Studies are currently underway to determine whether the heteroge-

Inhibitory effect (20 to 25\%) was not increased by increasing the concentration of meclofenamic acid 3-fold. Second, meclofenamic acid (10 to 30 $\mu$M) caused a competitive inhibition of the effects of PGE\textsubscript{1} (1.0 to 10 $\mu$M). Finally, meclofenamic acid at higher concentrations (30 to 100 $\mu$M) caused a nonspecific inhibition of the activation of cAMP synthesis by either PGE\textsubscript{1} or isoproterenol. We have concluded that even though meclofenamic acid has a complex set of effects on 1321N1 cells, it clearly is a potent inhibitor of the stimulation of cAMP synthesis by PGE\textsubscript{1}. In contrast, it should be pointed out that at least in other systems (17) indomethacin is a much more potent inhibitor ($K_{i}$ values in the low micromolar range) of prostaglandin synthesis than of PGE\textsubscript{1} action in 1321N1 cells ($K_{i}$ in the 100 to 200 $\mu$M range). The structural basis of the competitive component of the inhibition remains unclear.

These studies suggest that inhibition of PGE\textsubscript{1}-stimulated adenylyl cyclase may be an important facet of the inhibition by certain nonsteroidal anti-inflammatory agents of the physiological actions of PGE\textsubscript{1} and PGE\textsubscript{2}. Certainly, such a proposal is most convincing as an explanation of the actions of the mefenamates. Plasma levels attained in humans (17) for meclofenamic acid (1.35 $\mu$M), flufenamic acid (53 $\mu$M), and melen- 

acidic acid was 10 to 100 times more potent as an inhibitor of the effects of PGE\textsubscript{1} in 1321N1 cells than in the murine cell lines examined by Brunton et al. (16). It would seem important at this time to determine whether multiple classes of adenylyl cyclase-linked prostaglandin E receptors exist in nature in order to more coherently interpret the physiological significance of studies of individual cell lines.

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Stimulation of adenosine 3′:5′-monophosphate formation by prostaglandins in human astrocytoma cells. Inhibition by nonsteroidal anti-inflammatory agents.

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