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Activation of early events of the mitogenic response by a P_{2Y} purinoceptor with covalently bound 3′-*O*-(4-benzoyl)-benzoyladenosine 5′-triphosphate

(growth control/3′-*O*-(4-benzoyl)benzoyl-ATP/photoaffinity/arachidonate metabolism)

FERNANDO A. GONZALEZ*, DING-JI WANG, NING-NA HUANG, AND LEON A. HEPPEL†

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853

Contributed by Leon A. Heppel, September 24, 1990

ABSTRACT **3′-*O*-(4-Benzoyl)benzoyl-ATP (BzATP), a photoaffinity analog of ATP, was used as a ligand for a P_{2Y} purinoceptor (adenine nucleotide receptor) present in intact Swiss 3T3 and 3T6 cells and A-431 epidermoid carcinoma cells. Photolysis of serum-starved cells in the presence of 10–50 μM BzATP, followed by extensive washing to remove unincorporated BzATP, induced the release of arachidonic acid. A trace (<0.01%) of photoincorporated BzATP was as effective as when 50 μM BzATP or ATP was contained in the incubation medium during the assay. Photoincorporated BzATP also stimulated the production of prostaglandin E_2 and the accumulation of cyclic AMP. In previous studies, we demonstrated that these three events are obligatory early steps in a pathway leading to DNA synthesis in the above cell lines. The evidence indicated that the purinoceptor activated by extracellular ATP or BzATP was linked to a pertussis toxin-sensitive GTP-binding protein. Consistent with these observations, we now find that pertussis toxin inhibits the effect of photoincorporated BzATP on arachidonic acid release. These results indicate that BzATP is an effective agonist for the P_{2Y} purinoceptor concerned with stimulation of DNA synthesis in 3T3, 3T6, and A-431 cells. Furthermore, after photolysis it becomes irreversibly associated with intact cells and promotes the activation of early events required for DNA synthesis.**

Various growth factors, hormones, and neurotransmitters induce their characteristic effects upon binding to specific receptors on the plasma membrane of target cells (1). We recently have shown (2, 3) that extracellular ATP, when added in synergistic combination with some purified growth factors, stimulates cell growth in Swiss 3T3 and 3T6 mouse fibroblasts, A-431 epidermoid carcinoma cells, DDT_1MF-2 smooth muscle cells, human foreskin fibroblasts, BALB/MK mouse keratinocytes, and NIE-115 neuroblastoma cells. Mitogenic effects of ATP have also been reported for endothelial cells (4) and thymocytes (5). Extracellular ATP is believed to act by means of receptors for adenine nucleotides, called "purinoceptors" (for review, see ref. 6).

We have found that extracellular ATP, like many growth factors, activates a number of so-called "early events" of the mitogenic response in cultured cells. These early events include: (*i*) increase in cytosolic free calcium (7–9), (*ii*) formation of inositol phosphates and stimulation of calcium efflux (9, 10), (*iii*) release of arachidonic acid and formation of prostaglandin E_2 (PGE_2), (*iv*) formation of diacylglycerol and hydrolysis of phosphatidylcholine, (*v*) alkalinization of the cytosol, and (*vi*) activation of uridine uptake (unpublished results). Similar effects of ATP have been described by others in a variety of cell lines (11–20). Studies of various growth factors suggested that some early events were only regulatory for DNA synthesis while others were required for mitogenesis (for review, see ref. 1).

Of particular interest is the fact that stimulation of arachidonate metabolism seems to be a required step for ATP-dependent mitogenesis in 3T3, 3T6, and A-431 cells (N.-n.H., D.-j.W., F.A.G., and L.A.H., unpublished data). This pathway involves binding of ATP to a purinoceptor followed by activation of phospholipase A_2 to form arachidonic acid, which in turn is oxidized to PGE_2 by cyclooxygenase. PGE_2 is released into the medium and, by autocrine induction, stimulates adenylyl cyclase and increases the levels of cAMP. Elevation of cAMP levels acts as a mitogenic signal in our particular cell lines (see also ref. 1). Part of the evidence that the pathway is necessary for stimulation of DNA synthesis is as follows: ATP-dependent DNA synthesis was inhibited by pretreatment with pertussis toxin, which also inhibited arachidonic acid release and elevation of cAMP levels. These effects were completely overcome by exogenous PGE_2. Similarly, ATP-dependent DNA synthesis was greatly decreased by inhibitors of phospholipase A_2, which blocked arachidonic acid release, PGE_2 production, and cAMP accumulation. All of this was reversed by exogenous arachidonic acid and PGE_2. Similar data were obtained with cyclooxygenase inhibitors. Therefore, we decided to study the arachidonic acid pathway using a photoreactive analog of ATP, 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP).

BzATP has been successfully photoincorporated into a variety of ATP binding proteins including mitochondrial F_1-ATPase (21), sarcoplasmic reticulum Ca^{2+}/Mg^{2+}-ATPase (22), myosin ATPase (23), chloroplast coupling factor 1 (24), and tonoplast ATPase (25). Very recently Gonzalez *et al.* (26) showed that BzATP could activate a purinoceptor in 3T6 cells that promotes the formation of permeability lesions in the plasma membrane. Erb *et al.* (27) achieved the photoincorporation of BzATP into this receptor, using intact 3T6 cells. After removal of unbound BzATP and shifting the temperature to 37°C, the cells were found to be permeable to nucleotides. Similarly, BzATP has been photoincorporated into a P_{2Y} purinoceptor in a membrane preparation of turkey erythrocytes (28). In these cells, it promoted P_{2Y} purinoceptor-mediated, guanine nucleotide-dependent activation of phospholipase C.

The present study demonstrates that BzATP could be covalently bound to a P_{2Y} purinoceptor in intact, serum-starved, mammalian cells and thereby mediate a series of physiological responses necessary for ATP-dependent mitogenesis. This *in vivo* activity was inhibited by pertussis toxin,

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Abbreviations: BzATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; PGE_2, prostaglandin E_2; G protein, GTP-binding protein.

*Present address: Howard Hughes Medical Institute, Program in Molecular Medicine, Department of Biochemistry, University of Massachusetts Medical Center, Worcester, MA 01605.
†To whom reprint requests should be addressed.

which is consistent with other evidence for coupling to a GTP-binding protein (G protein).

MATERIALS AND METHODS

Cell Culture and Materials. Swiss 3T3 and 3T6 mouse fibroblasts and human A-431 epidermoid carcinoma cells were subcultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10 mM Hepes, 44 mM $NaHCO_3$, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 5% (3T6 and A-431) or 10% (3T3) (vol/vol) fetal bovine serum (GIBCO). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Cell-plating density was 1×10^5 cells in either 35-mm dishes or 12-multiwell Corning plates, and cells were used at confluence. ATP and UTP were from Boehringer Mannheim. Adenosine and BzATP were from Sigma. cAMP assay kits were purchased from Amersham. [5,6,8,11,12,14,15-^{3}H]PGE_2 and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid were from DuPont/New England Nuclear. PGE_2 antiserum was from Advanced Magnetics (Cambridge, MA). Pertussis toxin was from List Biological Laboratories (Campbell, CA). DMEM, Waymouth medium, and fetal bovine serum were obtained from GIBCO.

Photoincorporation of BzATP into Intact Cells. The cell cultures were washed twice with 1 ml of HBS buffer (10 mM Hepes, pH 7.4/140 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/10 mM glucose) kept at 20°C, and then 1 ml of the same buffer was added and the cell cultures were transferred to ice. After 3 min at 4°C, BzATP or ATP, as indicated in the figure legends, was added, and the cells were kept at 4°C in the dark for 15 min. The control cultures were kept at 4°C in the dark for an additional 15 min, while the experimental cultures were irradiated with long-wavelength UV light from a UVL-56 lamp positioned 3–5 cm above the cells, with a Pyrex glass filter covering the dishes. After irradiation, the cells were washed four times with 1 ml of ice-cold HBS buffer to remove unincorporated BzATP, and 1 ml of HBS buffer (37°C) was added. These cultures were incubated at 37°C for the time required and were used to measure arachidonic acid release, prostaglandin E_2 production, or cAMP accumulation, as described in the figure legends.

RESULTS

Photoincorporation of BzATP Induces the Release of Arachidonic Acid. Addition of ATP or its photoreactive analog, BzATP, stimulated the release of [^{3}H]arachidonate from prelabeled, serum-starved Swiss 3T6 cells (Fig. 1*A*). Onset of the effect was rapid, a significant stimulation of release being observed by 3 min after addition of either nucleotide and a maximal effect being observed by 12 min. The two nucleotides displayed similar activity at a concentration of 50 μM. Fig. 1*B* shows the effect on preloaded cells of adding 50 μM BzATP, followed by photolysis at 4°C, extensive washing, and subsequent incubation at 37°C. This treatment caused release of [^{3}H]arachidonic acid with similar kinetics to that seen in Fig. 1*A*. With highly radioactive BzATP, we noted that only a faint trace (<0.01%) of the nucleotide remained cell-bound after the wash step, but this sufficed to activate the P_{2Y} purinoceptor.

Photolysis was necessary because little or no stimulation of [^{3}H]arachidonic acid release occurred if the following sequence of steps, BzATP addition, temperature shifts, and washings, was carried out in the absence of ultraviolet light (Fig. 2*B*). ATP was inactive (Fig. 2*A*, lane c), but it was a potent inhibitor of [^{3}H]arachidonic acid release mediated by BzATP (Fig. 3). There was no inhibition by UTP or adenosine when mixed in 10-fold excess with BzATP in a photolysis experiment (Fig. 3). Finally, when the cells were first irradiated and this treatment was followed by incubation with either ATP or BzATP in the usual way, a normal response was obtained (Fig. 2*A*, lanes d and e).

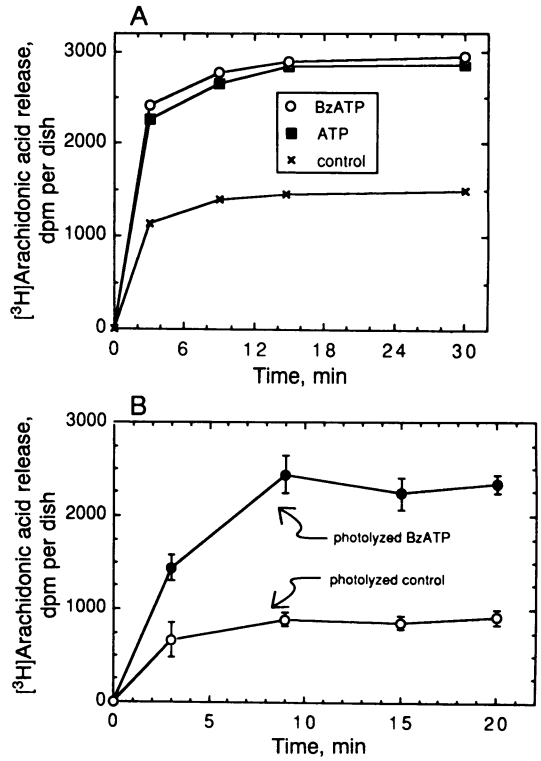

FIG. 1. Time course of [^{3}H]arachidonic acid release in 3T3 cells stimulated by ATP or BzATP added to the medium or by photoincorporated BzATP. (*A*) Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated for 24 hr in the absence of serum in 1.0 ml of DMEM/Waymouth medium, 1:1 (vol/vol), containing 0.1 μCi of [^{3}H]arachidonic acid. Prior to the initiation of the experiment, the media were aspirated and the cells were washed three times at half-min intervals with DMEM/Waymouth medium, followed by 1.5 ml of the same medium either without additions or with 50 μM ATP or 50 μM BzATP. The cells were incubated at 37°C, and samples were removed at intervals for measurement in a liquid scintillation counter. The experiment was performed three times. The maximum variation from the mean was less than 10% for all points. (*B*) The quiescent cultures were washed and incubated with DMEM/Waymouth as above. Photoincorporation of BzATP was carried out as described except that the final incubation at 37°C was in 1.5 ml of HBS buffer. Samples were removed at intervals for counting by liquid scintillation spectrophotometry. The experiment was carried out three times with similar results.

Accumulation of cAMP Is Stimulated by Photoincorporation of BzATP. In a previous study, we demonstrated that extracellular ATP elevates cAMP levels in 3T3, 3T6, and A-431 cells in a concentration-dependent manner, with a maximal effect at 50 μM. This concentration also corresponded to the maximal stimulation of DNA synthesis (N.n.H., D.j.W., F.A.G., and L.A.H., unpublished data). Accumulation of cAMP occurred after an initial delay period of 15 min and reached a maximum after about 45 min of incubation in the presence of ATP. Fig. 4 shows that photoincorporation of BzATP stimulated cAMP accumulation in A-431 cells. Similar results were obtained when 3T3 and 3T6 cells were used (not shown). No stimulation was seen with ATP in a similar experiment. Furthermore, a 2-fold excess of ATP prevented the enhancement of cAMP accumulation seen with BzATP alone. These experiments were carried out in the absence of RO-20-1724, a cAMP phosphodiesterase inhibitor that would have increased cAMP accumulation in all of the dishes.

Photoincorporated BzATP Stimulates PGE_2 Production. The delayed accumulation of cAMP in 3T3, 3T6, and A-431

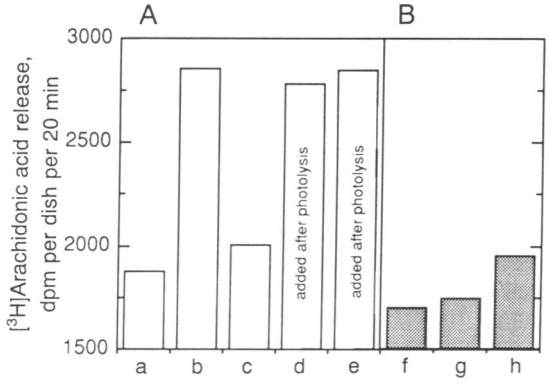

FIG. 2. Effect of photoincorporation of BzATP on [^{3}H]arachidonic acid release in 3T6 cells. Experimental conditions were as in Fig. 1*B* except that [^{3}H]arachidonic acid release was measured after a single incubation period of 20 min. (*A*) Ultraviolet exposure was carried out. Lanes: a, no additions; b, stimulation of [^{3}H]arachidonic acid release by photoincorporation with 10 μM BzATP, followed by removal of unbound BzATP; c, no significant effect of 10 μM ATP after the same treatment; d and e, stimulation of [^{3}H]arachidonic acid release by both ATP and BzATP if added after exposure of cells to ultraviolet light. (*B*) In lanes f, g, and h, treatment was as for lanes a, b, and c in *A*, but there was no exposure to ultraviolet light. The arachidonic acid release experiments shown in Figs. 2, 3, and 6 were carried out three to four times with similar results but quantitative variation. Pooled data for three cell lines showed the following release of [^{3}H]arachidonic acid after photoincorporation of BzATP, expressed as a percent of controls that were photolyzed in the absence of BzATP: 3T3 cells, 189 ± 28% (n = 6); 3T6 cells, 161 ± 26% (n = 5); and A-431 cells, 179 ± 22% (n = 4). Increasing the concentration of BzATP from 10 to 50 μM did not change results significantly.

cells, caused by extracellular ATP, was explained by demonstrating that stimulation of arachidonic acid release was followed by PGE_2 production (N.n.H., D.j.W., F.A.G., and L.A.H., unpublished data). Fig. 5 shows that photoincorporation of BzATP in A-431 cells also resulted in the production of PGE_2. In the presence of a 10-fold excess of ATP, this effect of BzATP was completely blocked. Similar results were obtained with 3T3 and 3T6 cells (not shown).

Preincubation with Pertussis Toxin Inhibits the Effect of Photoincorporated BzATP on [^{3}H]Arachidonic Acid Release. Pertussis toxin inhibited ATP-stimulated arachidonic acid release and PGE_2 synthesis in 3T3, 3T6, and A-431 cells, suggesting the involvement of a pertussis toxin-sensitive G protein. Further evidence was provided by the fact that, in permeabilized cells, guanosine 5′-[β-thio]diphosphate inhibited, while guanosine 5′-[γ-thio]triphosphate enhanced arachidonic acid release caused by ATP (N.n.H., D.j.W., F.A.G., and L.A.H., unpublished data). Fig. 6 shows that pretreatment with pertussis toxin blocked the release of [^{3}H]arachidonic acid stimulated by photoincorporated

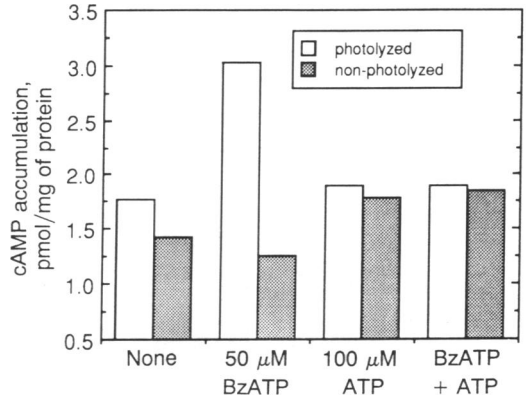

FIG. 4. Effect of photoincorporation of BzATP on cAMP accumulation in A-431 cells. Confluent A-431 cells were starved in DMEM/Waymouth medium 1:1 (vol/vol), for 20 to 24 hr. The photoincorporation of BzATP was performed as described. The cultures were incubated for 40 min at 37°C, after which the medium was rapidly aspirated and the cAMP content of the cell monolayer was determined with a cAMP assay kit (Amersham) as described by the supplier. The accumulation of cAMP after photoincorporation of 50 μM BzATP, expressed as a percent of controls photolyzed in the absence of BzATP, was 170 ± 20% (n = 3).

BzATP in A-431 cells. Similar results were obtained with 3T6 cells (data not shown).

DISCUSSION

BzATP was introduced by Williams and Coleman (21) as a new photoaffinity probe for exploring the nucleotide binding sites on mitochondrial F_1-ATPase from rat liver. It has a number of advantages over previously available probes (29, 30). (*i*) BzATP can be photoactivated by relatively nondestructive, long-wavelength UV light in contrast to the high-energy, short-wavelength UV light needed for other probes. (*ii*) A low-energy, diradical, triplet intermediate is formed that does not react with water but preferentially abstracts hydrogen from carbon–hydrogen bonds. (*iii*) The activated state has a relatively long lifetime.

In collaborative studies with the laboratory of G. A. Weisman (26, 27, 31), it was found that, in the dark, BzATP increased the plasma membrane permeability of transformed 3T6 mouse fibroblasts to normally impermeant, hydrophilic molecules, as previously found for ATP (32, 33). For permeabilization, BzATP behaved in many respects like ATP itself, and it was active at lower concentrations. Both nucleotides required an alkaline pH and low levels of divalent cations. Sealing occurred at neutral pH in the presence of equimolar divalent cations. By shifting from sealing to permeabilizing conditions and making a fresh addition of nucleotide, the cells could be made permeable once more, and the cycle of permeabilization and sealing could be repeated

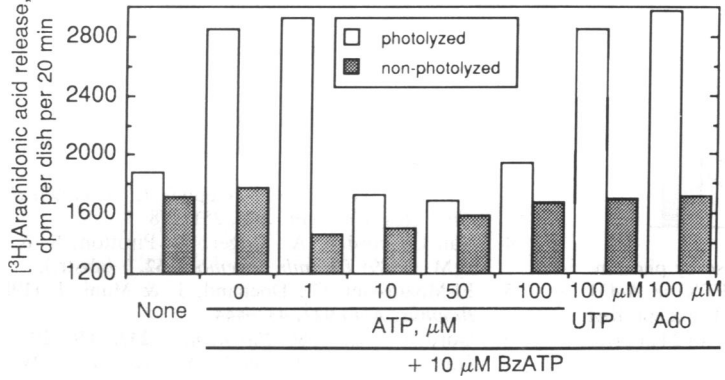

FIG. 3. Stimulation of [^{3}H]-arachidonic acid release by BzATP in 3T6 cells was inhibited by ATP but not by UTP or adenosine. Experimental conditions were as in Fig. 2*A*.

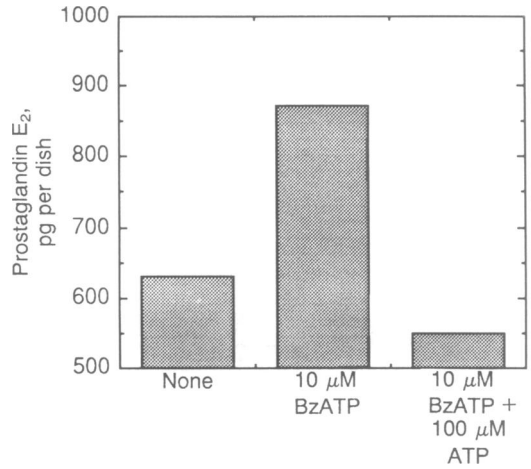

FIG. 5. Stimulation of PGE_2 production by photoincorporation of BzATP in A-431 cells. Experimental conditions were as in Fig. 4 except that PGE_2 production was measured with a 100-μl aliquot of the medium after the 40-min incubation at 37°C in HBS buffer. A radioimmunoassay was carried out by using anti-PGE_2 serum from Advanced Magnetics and following supplier instructions. Prostaglandin formation after photoincorporation of 10 μM BzATP, expressed as a percent of controls photolyzed in the absence of BZATP, was 133 ± 9% (n = 3).

several times. Photoincorporation of BzATP under UV light also increased plasma membrane permeability except that it was irreversible. Now the cells could be subjected to repeated cycles of permeabilization and sealing simply by changes of pH and concentration of divalent cations. No repeated additions of BzATP were necessary.

In the present investigation, we have shown that BzATP can be covalently bound to a P_{2Y} purinoceptor in intact, serum-starved 3T3, 3T6, and A-431 cells under UV light and thereby can mediate arachidonic acid release, PGE_2 synthesis, and a delayed elevation in cAMP levels. The designation "P_{2Y}" is based on receptor specificity for ATP and ADP (34). These early responses appeared to be obligatory for DNA synthesis in these cell lines, as pointed out above. In the present study, it also was shown that pertussis toxin inhibited the release of arachidonic acid that was stimulated by covalently incorporated BzATP, suggesting the involvement of a pertussis toxin-sensitive G protein. This observation is consistent with earlier results showing that, in permeabilized cells, guanosine 5′-[β-thio]diphosphate inhibited while guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) enhanced arachidonic acid release stimulated by extracellular ATP (N.n.H., D.j.W., F.A.G., and L.A.H., unpublished data). Working

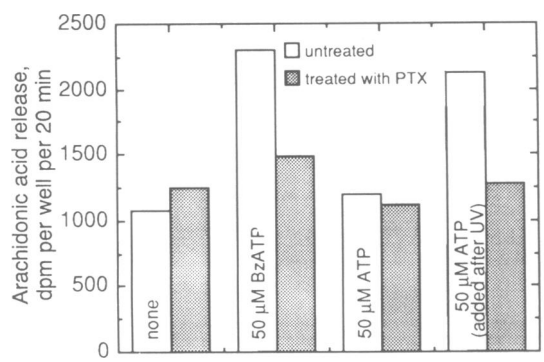

FIG. 6. Stimulation of [^{3}H]arachidonic acid release by photoincorporated BzATP is inhibited by pertussis toxin (PTX) in A-431 cells. Experimental conditions were as in Fig. 2*A* except that pertussis toxin at 25 ng/ml was incubated with the cells for 24 hr prior to addition of BzATP or ATP.

with isolated plasma membranes, Boyer and Harden (28) found that the phospholipase C response to submaximally effective concentrations of GTP[γ-S] was markedly increased in turkey erythrocyte membranes previously photolyzed in the presence of BzATP. In a later publication (35), evidence for receptor labeling by [^{32}P]BzATP was presented.

We have recently demonstrated other effects of photoincorporated BzATP (unpublished results). It stimulated arachidonic acid release and cAMP accumulation in DDT_1MF-2 smooth muscle cells. Both processes were inhibited by ATP, as noted here for 3T6 cells (Fig. 3). In A-431 cells, photoincorporated BzATP stimulated uridine uptake—one of the early signals seen with a number of mitogens (1).

Previous work suggests that BzATP serves as an agonist for at least three different ATP receptors present in 3T6 mouse fibroblasts. One of these is a purinoceptor whose activation causes an increase in plasma membrane permeability for Na^+ and K^+ and subsequently for nucleotides and other normally impermeant small molecules. It is highly specific for ATP and a number of ATP analogues (26, 27, 32, 33, 36). A second receptor mediates Ca^{2+} mobilization and inositol phosphate formation (7–10). It displays a broader nucleotide specificity. Both ATP and UTP are good agonists, but ADP is inactive. In addition to differences in agonist specificity, these two receptors can be distinguished by differences in ease of desensitization and by the behavior of a cell variant (36). A third receptor, a P_{2Y} purinoceptor most active with ATP and ADP, is concerned with certain "early events" and with DNA synthesis.

Preliminary experiments have revealed another effect seen with photoincorporated BzATP (D.j.W., N.n.H., L.A.H., and I. Friedberg, unpublished data). Prolonged incubation in the presence of ATP or BzATP desensitized both 3T6 and DDT_1MF-2 cells so that subsequently they failed to respond to either 50 μM ATP or 50 μM BzATP. The responses being examined were arachidonic acid release and cAMP accumulation. We found that these same responses were also blocked when photoincorporation experiments with BzATP were carried out with cells that had been preincubated for a prolonged period with ATP or BzATP.

A P_{2Y} purinoceptor that stimulates arachidonic acid metabolism and DNA synthesis has not been purified and characterized. Membranes from DDT_1MF-2 cells represent a rich source and have a further advantage. They lack the receptor for ATP-dependent permeabilization, which also binds BzATP and which could confuse efforts to measure the content of P_{2Y} purinoceptors.

We are grateful to Mrs. Sharon Johnston for expert tissue culture assistance and to Ms. Vicki Shaff for secretarial assistance. Special thanks are due to Dr. E. Rozengurt for providing 3T3 and 3T6 cells on repeated occasions. Cultures from his laboratory have shown consistently high responsiveness to P_2 purinoceptor agonists. This work was supported by grants from the National Institutes of Health (DK-11789), the National Institute of Aging (AG-07429), and the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office, and the National Science Foundation.

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