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PRESENCE OF BOTH A₁ AND A_{2a} ADENOSINE RECEPTORS IN HUMAN CELLS AND THEIR INTERACTION

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SUMMARY

We have obtained pharmacological evidence for the expression of both A₁- (inhibitory) and A_{2a}- (stimulatory) adenosine receptors in cultured human foreskin fibroblasts and lung fibroblasts. The A₁ receptors were sufficiently abundant in foreskin fibroblasts so that binding studies with a radioactively labeled specific ligand confirmed their existence. Both receptors were activated during the stimulation of cAMP accumulation and DNA synthesis by adenosine.

Adenosine activates specific cell surface receptors, which there upon regulate a variety of physiological functions. Adenosine receptors were originally classified by Burnstock [reviewed in (1)] as P₁ to distinguish them from P₂ receptors which interact mainly with ATP. Later, the P₁-adenosine receptors were further subdivided into A₁ and A₂ receptors based on selectivity for a series of adenosine analogues (2, 3). Demonstration of adenosine receptors in many types of cells has been made primarily by functional assays. Thus, it was found that A_{2a}-adenosine receptors stimulated adenylyl cyclase, while A₁ receptors inhibited this enzyme (1). This occurs by way of coupling to G_i (inhibitory) or G_s (stimulatory), intermediary ATP-binding proteins. Today it is realized that A₁-adenosine receptors also stimulate other functions such as the opening of K⁺ channels (1).

There have been a few reports in which it was found that a single cell coexpresses both A₁- and A_{2a}-adenosine receptors (1, 3, 4). The best documented case concerns the DDT₁-MF2 smooth muscle cell line (3). In the present investigation, we obtained pharmacological evidence for the expression of both A₁- and A_{2a}-adenosine receptors in the FS4 cell line derived in 1973 from human foreskin tissue (5), in cultures of human foreskin fibroblasts isolated within the past year, and in IMR90 human lung fibroblasts (6). The data suggest that both A₁ and A_{2a} receptors are activated during the stimulation of cAMP accumulation and DNA synthesis by adenosine in foreskin fibroblasts.

MATERIALS AND METHODS

Materials

Nucleotides, insulin and adenosine were from Boehringer Mannheim. Pertussis toxin was obtained from List Laboratories, Campbell, CA. Epidermal growth factor (EGF) was obtained from Upstate Biotechnology, Inc. [³H]Thymidine and cAMP assay kits were

purchased from Amersham Corporation. Culture media and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. Other reagents were purchased from Sigma.

Cell culture

FS4 cells were kindly supplied by Dr. J. Vilcek of New York University Medical Center. Human foreskin fibroblast cells were obtained from Advanced Biotechnologies, Inc., Columbia, MD. Human embryo lung fibroblasts (IMR90, 22–26 doublings) were from the Coriell Institute for Medical Research, Camden, NJ. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) as described (6, 7).

DNA synthesis

Quiescent cultures were washed with a 1:1 mixture of DMEM and Waymouth's medium and incorporation of [^3H]thymidine (0.25 $\mu\text{Ci}/\text{ml}$, 1 μM) was performed as described (7). After a 40 h incubation at 37°C, acid-insoluble radioactivity was measured. A 24-h incubation before addition of isotope ("serum starvation") was not necessary to reveal stimulation by ATP or adenosine, but this treatment made the cells more responsive to LPA.

Measurement of cAMP accumulation

Quiescent cultures were washed and incubated at 37°C for 2–3 h with a 1:1 mixture of DMEM and Waymouth's medium. RO20-1724 (10 μM) was added 5 min before other required additions except for Fig. 2. After 15 min, or as indicated, at 37°C, intracellular cAMP accumulation was measured with a binding protein assay kit obtained from Amersham Corporation.

Assay: Radioligand binding

Membranes prepared as described (3) were tested in radioligand binding assays (8) for the presence of A_1 and A_{2a} receptors, using [^3H]CPX and [^3H]CGS21680, respectively. Membranes were resuspended in 50 mM Tris (pH 7.4), 10 mM MgCl_2 , 0.01% CHAPS, and 100 μl membrane solution was used for saturation binding to A_1 receptors; the membrane concentration was 0.2 mg protein per ml. Appropriate [^3H]CPX (109 Ci/mmol) was added and the mixture was incubated at 37°C for 1 h. Two units/ml adenosine deaminase were added. NECA (final, 400 μM) was used as a non-specific reagent. Binding was terminated by rapid filtration with the same ice-cold buffer, using a Brandel cell harvester and Whatman GF/B, glass fiber filters.

RESULTS

For all results, similar data were obtained with each of the three cell types. Thus, adenosine shares with ATP, ADP and AMPPNP the distinction of being an unusually effective mitogen when presented to FS4, HFF or IMR90 cells in the absence of other growth factors. The stimulation of [^3H]thymidine incorporation was greater than seen for EGF or fetal bovine serum (Fig. 1). The unusually good mitogenic activity for these adenine derivatives was reported for another primate line, the African green monkey kidney epithelial cells (BSC-1) (9). Other evidence for mitogenic stimulation by adenosine included increases in percent

nuclear labeling by [³H]thymidine, chemical assay for cellular DNA using the bisbenzamide reaction (13), and cell counts (data not shown).

LPA, a mitogen of great current interest (7), synergizes with adenosine or ATP. In HFF cells, incorporation with [³H]thymidine was stimulated 13.5 fold by 50 μ M adenosine, 3.7 fold by 20 μ M LPA, and 29 fold by combined adenosine and LPA. No other pairs of mitogens were as effective.

Evidence for A_{2a}-adenosine receptors

We observed dose-dependent accumulation of cAMP when FS4 cells were incubated with adenosine (Fig. 2), or with the analogue, APEC, which is a highly specific agonist for A_{2a} receptors (Fig. 3). The specific antagonist for A_{2a} receptor binding, CSC, reduced intracellular cAMP levels that had been elevated by APEC (Fig. 4). CSC also reduced the accumulation of cAMP that had been stimulated by adenosine (Fig. 5). In addition, prolonged (24 h) preincubation of FS4 cells with APEC caused partial reduction of cAMP accumulation stimulated by adenosine, presumably due to selective desensitization of the A_{2a}-adenosine receptor (Fig. 2). Further evidence for the presence of A_{2a}-adenosine receptors in FS4, HFF and IMR90 cells is provided by the fact that pretreatment with cholera toxin (CTX) enhanced the elevation of cAMP levels produced by incubation with adenosine or APEC. In a typical experiment, similar to that described in Fig. 2, pmoles of cAMP per mg protein, formed in 15 min, were: Basal, 14; CTX, 50 ng/ml, 200; adenosine, 25 μ M, 50; adenosine plus CTX, 1200.

Presence of A₁-adenosine receptors expressed in FS4, HFF and IMR90 cells

The presence of A₁ receptors in human cells was demonstrated by finding partial inhibition of cAMP accumulation caused by isoproterenol or forskolin, in the presence of the A₁ agonists R-PIA or CPA. The inhibition induced by R-PIA or CPA was overcome by having the specific A₁ antagonist, CPX, also present. For example, cAMP accumulation (pmoles/mg protein), in an experiment similar to Fig. 2, was as follows: Basal, 53; 0.1 μ M R-PIA, 48; 10 μ M isoproterenol, 695; isoproterenol plus R-PIA, 520; isoproterenol plus R-PIA plus 50 nM CPX, 660. In addition to the specific antagonist, CPX, pretreatment with pertussis toxin (which inactivates *G_{ia}*) also overcame the effect of R-PIA.

Radioligand binding in FS4 and HFF cell membranes

In saturation experiments with membranes from FS4 and HFF cells, there was considerable [³H]CPX binding (for FS4, *n* = 1; *K_a* = 21 nM; $\beta_{\max}$ = 1.1 pmole/mg protein). However, in both FS4 and HFF cell membranes, there was no detectable specific binding to A_{2a} receptors. Thus, the binding data provide additional evidence for A₁-adenosine receptors only.

Evidence for an interaction between the A₁- and A_{2a}-adenosine receptors during adenosine-stimulated [³H]thymidine incorporation

Fig. 7 shows that prolonged (24 h) preincubation of FS4 cells with the A₁ agonists CPA and R-PIA, or A_{2a} agonist APEC, partially reduced the mitogenic response to adenosine. A partial reduction was also obtained after a brief (1 h) exposure of FS4 cells to 10 μ M NECA,

conditions reported to cause complete desensitization of A_{2a} receptors in another cell line (10). In addition, adenosine-stimulated DNA synthesis was inhibited as follows in the presence of specific A_1 - or A_{2a} -adenosine receptor antagonists: 50% by 10 nM or 25 nM CPX; 60–70% by 1 nM CSC. The data suggest that both A_1 - and A_{2a} -adenosine receptors are involved in the stimulation of DNA synthesis. In contrast to results with 3T3 cells (11), NECA was not able to replace adenosine as a mitogen.

Evidence for an interaction between A_1 - and A_{2a} -adenosine receptors during adenosine-stimulated cAMP accumulation

Elevation of cellular cAMP levels induced by adenosine was inhibited by either the A_1 -receptor antagonist, CPX, or the A_{2a} -receptor antagonist, CSC (Fig. 5). This indicates that the A_1 and A_{2a} receptors interacted in some way during adenosine-stimulated cAMP accumulation. Additional evidence for interaction (see Fig. 2) is the fact that prolonged preincubation with CPA or R-PIA, specific A_1 -adenosine agonists, caused partial inhibition of adenosine-dependent elevation in cAMP levels, while APEC, an A_{2a} -adenosine agonist, had the same effect. The possible nature of this interaction is discussed below.

DISCUSSION

We have demonstrated the presence of A_1 - as well as A_{2a} -adenosine receptors in human diploid foreskin fibroblasts and in human diploid lung fibroblasts. The evidence is based mainly on pharmacological experiments with specific A_1 and A_{2a} agonists and antagonists. In addition, binding of a radioactively labeled A_1 -receptor agonist to FS4 and HFF was measured.

In this paper, we have also presented evidence for an interaction between the A_1 - and A_{2a} -adenosine receptors during adenosine-stimulated cAMP accumulation. The results can best be explained by a model proposed by Tang and Gilman (14). According to this model, G_{sa} (activated when adenosine binds to A_{2a} receptors) interacts with $\beta\gamma$ (released in high concentration when adenosine binds to A_1 receptors). The interaction causes enhanced stimulation of adenylyl cyclase II (or IV), greater than achieved by G_{sa} alone. As explained in reference (15), agonists acting through “inhibitory” (A_1) receptors coupled to G_i are converted into stimulators of cAMP synthesis. The fact that cAMP accumulation by G_{sa} is greatly potentiated by forskolin (16) is consistent with the presence of adenylyl cyclase II, and this is shown in Fig. 4. This type of interaction between G_{sa} and $\beta\gamma$ was first observed by Tang and Gilman (14) and shown to occur in transfected intact cells in Bourne’s laboratory (15).

As shown in Fig. 6, prior incubation with pertussis toxin caused substantial elevation in cAMP accumulation stimulated by APEC in FS4 cells. We interpret this result to mean that pertussis toxin ADP-ribosylates G_i (and/or G_0), with decrease in the affinity of the α subunit for the $\beta\gamma$ complex, as demonstrated by Casey et al. (12) and by Katoda et al. (13). The $\beta\gamma$ complex interacts with G_{sa} , activated by adenosine or APEC bound to A_{2a} receptors, to cause enhanced accumulation of cAMP, greater than seen in the absence of pretreatment with pertussis toxin. The experiment shown in Fig. 6 has been repeated many times with all three cell types and with NECA, APEC, adenosine, or ATP as a source of G_{sa} .

Enhanced accumulation of cAMP by interaction of G_{sa} with $\beta\gamma$ was also seen in ongoing experiments in which human cells were incubated with the specific A_{2a} -receptor agonist, APEC, together with the A_1 -adenosine receptor agonist, R-PIA. Even greater enhancement was seen with the combination of NECA and R-PIA (or CPA). Finally, we postulate that stimulation of cAMP accumulation by adenosine itself was the sum of a cAMP elevation due to activation of G_{sa} after A_{2a} -receptor binding, plus an augmentation caused by G_s interacting with $\beta\gamma$ (released by activated G_i). This accounts for the inhibition produced either by an A_1 antagonist or an A_{2a} antagonist (Fig. 5). A similar explanation accounts for the partial inhibition of adenosine-dependent cAMP accumulation in FS4 cells after desensitization of A_1 receptors with prolonged exposure to R-PIA or CPA (Fig. 2). The augmentation due to interaction of G_{sa} with $\beta\gamma$ is presumed to be lost by the desensitization. We ignore possible effects of G_{ia} , reported to be weak.

Current experiments involve transfection with the α subunit of transducin (15), or experiments using a fusion protein which is a specific probe that acts as a scavenger for $\beta\gamma$ subunits (17). These experiments can provide direct evidence for participation of $\beta\gamma$ in adenosine-dependent stimulation of cAMP accumulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ADO	adenosine
APEC	2-[(2-aminoethylamino)carbonyl-ethylphenylethylamino]-5'-N-ethylcarboxamidoadenosine
CPA	N-cyclopentyl-adenosine (A_1 agonist)
CPX	DPCPX, 8-cyclopentyl-1,3-dipropylxanthine (A_1 antagonist)
CSC	8-(3-chlorostyryl)caffeine (A_{2a} antagonist)
FSK	forskolin
LPA	lysophosphatidic acid
NECA	N-ethylcarboxamidoadenosine (A_2 agonist)
PTX	pertussis toxin
RO20-1724	inhibitor of cAMP phosphodiesterase

R-PIA R-phenylisopropyladenosine (A_1 agonist). The adenosine analogues are described in great detail by Jacobson et al. (Drug Development Research 28, 226–231, 1993)

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Fig. 1. Stimulation of [^3H]thymidine incorporation in quiescent FS4 cultures. Procedure as in “Methods”. AMPNP, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; EGF, epidermal growth factor.

Fig. 2.

Concentration dependence for stimulation of cAMP accumulation by adenosine (Curve 1) and the effects of receptor desensitization. FS4 cells in 12-well clusters were washed and incubated at 37°C for 45 min with 1:1 DMEM:Waymouth medium containing adenosine as indicated. Cellular cAMP measured as in "Methods". Curve 1, control. Curves 2–5: Cells were pretreated for 24 h with 100 nM R-PIA (Curve 2); 100 nM CPA (Curve 3); 50 nM APEC (Curve 4); 25 μM ADO (Curve 5). After washing, adenosine was added as in Curve 1.

Fig. 3.
Concentration dependence for stimulation of cAMP levels by the A_{2a} -adenosine receptor agonist, APEC, in FS4 cells. Conditions as in legend to Fig. 2.

Fig. 4. Effect of 10 nM CSC, an A_{2a} -adenosine receptor antagonist, on cAMP accumulation stimulated by 100 nM APEC, an A_{2a} agonist. Conditions as in Fig. 2. Forskolin (1 μ M) caused a huge synergistic augmentation of the effect of 100 nM APEC on cAMP levels, suggesting that FS4 cells contain adenylyl cyclases II or IV (16).

Fig. 5. Effect of 25 nM CPX and 10 nM CSC, an A₁- and A_{2a}-adenosine receptor antagonist, respectively, on cAMP accumulation stimulated by 25 µM adenosine. Conditions as for Fig. 2.

Fig. 6.

Effect of pretreatment with pertussis toxin (PTX) on the stimulation of cAMP accumulation by APEC, an A_{2a} -adenosine receptor agonist. FS4 cells were incubated for 4 h in 1:DMEM:Waymouth medium with 50 ng/ml PTX. After washing, the cells, along with untreated controls, were assayed as usual for cAMP accumulation, using 100 nM APEC to stimulate them.

Fig. 7.

Partial inhibition of adenosine-stimulated [^3H]thymidine incorporation after desensitization by A_1 and A_{2a} receptor agonists, and greater desensitization by ADO. FS4 cells were preincubated for 24 h at 37°C with 100 nM R-PIA or CPA (Curves 3 and 4), or 50 nM APEC (Curve 2), or 25 μM adenosine (Curve 5), and the control (Curve 1). The cells were washed and [^3H]thymidine incorporation was measured in the presence of different levels of adenosine (see “Methods”).