Stimulation of human umbilical vein endothelial cell proliferation by A₂-adenosine and β ₂-adrenoceptors

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1 Adenosine is known to stimulate capillary outgrowth and endothelial cell proliferation, but the underlying mechanism has not been identified. In order to identify the receptor subtype involved, the effects of adenosine receptor agonists and antagonists on human umbilical vein endothelial cell (HUVEC) proliferation were investigated.

2 Raising intracellular adenosine levels by use of the adenosine transport inhibitor, 4-nitrobenzylthioinosine (NBMPR) did not affect cell growth. This observation suggests that stimulation of an extracellular adenosine receptor generates the mitogenic signal.

3 In the presence of adenosine deaminase (ADA), which was used to remove adenosine present in the culture medium, the adenosine receptor agonists N-ethylcarboxamidoadenosine (NECA, non-selective) and CGS21680 (A_{2A}-receptor-selective) stimulated [³H]-thymidine incorporation with a half-maximum effect at about 10 nM, while N⁶-cyclopentyladenosine (CPA, A₁-selective) was about 100 fold less potent. The adenosine receptor antagonist, xanthine amine congener (XAC) produced a concentration-dependent decrease in endothelial cell proliferation with a half-maximum effect at about 10 nM. Hence, stimulation of an endothelial A_{2A}-adenosine receptor seems responsible for the mitogenic signal.

4 In the presence of ADA, isoprenaline is also able to stimulate [³H]-thymidine incorporation with a half maximal effect of about 3 nM, an effect, which is reversed by the highly β ₂-selective antagonist, ICI 118,551. In the absence of ADA, isoprenaline exerts only a minor stimulatory effect. Combination of A_{2A} adenosine and β ₂-adrenoceptor agonists did not further enhance [³H]-thymidine incorporation when compared to the sole addition of each agonist. We therefore conclude that both receptors stimulate endothelial cell proliferation via a common signal transduction pathway.

5 Both receptors are coupled to stimulation of adenylyl cyclase via the stimulatory G protein G_s. However, *direct* activation of downstream effectors in the cyclic AMP-signalling cascade (G_s with cholera toxin, adenylyl cyclase with forskolin, protein kinase A with ⁸Br-cyclic AMP) not only failed to mimic the action of receptor-activation, but even reduced cell proliferation.

6 Similarly, pertussis toxin-treatment which inactivated the G_{i2} protein present in HUVEC and thus inhibited cell proliferation *per se*, did not impair the ability of A_{2A}-receptor agonists to stimulate cell proliferation. This suggests that the A_{2A}-adenosine and β ₂-adrenoceptor-mediated stimulation of endothelial cell proliferation occurs via a mechanism that is independent of G_s and G_i.

Keywords: Human umbilical vein endothelial cells (HUVEC); adenosine; β -adrenoceptor; cyclic AMP; proliferation

Introduction

Endothelial cell proliferation and angiogenesis occur in response to a variety of physiological and pathophysiological stimuli. As the initial response, the endothelial cells secrete proteolytic enzymes which results in fragmentation of the basal lamina; subsequently, the endothelial cells migrate through the fragmented lamina into the perivascular space where they proliferate and form new capillaries (Folkman & Shing, 1992). In the last decade, several mitogenic factors have been identified that stimulate endothelial cell proliferation and neo-vascularization; in particular, fibroblast growth factors, endothelial cell growth factor, transforming growth factors and angiogenin have been identified as important mitogens (Folkman & Shing, 1992; Bobik & Campbell, 1993). Hypoxia is also known to be a strong stimulus for angiogenesis, e.g. in processes like tumour growth, diabetic retinopathy, wound healing and collateralisation. Adenosine, which has long been known as a potent vasodilator (Berne, 1963), is released by hypoxic tissue in large amounts and findings on different animal models additionally suggest this nucleoside as an endothelial mitogen linking the altered

metabolism in oxygen-deprived cells to the formation of new capillaries (Ziada *et al.*, 1984; Dusseau *et al.*, 1986). In the meantime, a mitogenic effect of adenosine in cultured endothelial cells has been verified by several authors (Meininger & Granger, 1990; Van Daele *et al.*, 1992; Ethier *et al.*, 1993), but the site as well as the mechanism of action of the nucleoside remain unclear at present (Folkman & Shing, 1992; Collis & Hourani, 1993). Van Daele *et al.* (1992) found only a mitogenic effect for adenosine itself and for ATP, but no effect for typical adenosine receptor ligands. Hence, they concluded that adenosine may act via an intracellular site and ATP via a P₂-purinoceptor on the plasma membrane. In contrast, other workers reported adenosine-induced endothelial cell proliferation which is mimicked by adenosine receptor agonists and blocked by antagonists, thus implying rather an extracellular adenosine receptor-mediated site of action (Meininger & Granger, 1990; Ethier *et al.*, 1993); however, there is still no information with respect to the responsible adenosine receptor subtype, the type of the G protein involved in signal transduction and the nature of the intracellular messenger.

In the present study, we have therefore determined (*i*) which adenosine receptor subtype (A₁, A_{2A}, A_{2B}, or A₃)

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mediates the mitogenic action of adenosine on the endothelium and whether endothelial cell growth is also affected by changes in intracellular adenosine levels; (ii) which G protein (G_s , a subtype of G_i or neither) is involved in transduction of the mitogenic signal; and (iii) the role of adenosine 3':5'-cyclic monophosphate (cyclic AMP). The findings obtained with adenosine analogues were compared with those obtained for isoprenaline, since the β -adrenoceptor is also a prototypical G_s -coupled receptor known to reside on endothelial cells. All experiments were carried out on human umbilical vein endothelial cells (HUVEC).

Methods

Cell culture

HUVEC were isolated according to Jaffe *et al.* (1973). Cords were rinsed twice with phosphate-buffered-saline (PBS) and then incubated for 20 min with 0.2% collagenase in PBS. The cell suspension was collected, centrifuged and the cell pellet was resuspended in medium 199 enriched with endothelial cell growth supplement (ECGS), $100 \mu\text{g ml}^{-1}$ streptomycin, 100 u ml^{-1} penicillin, $0.25 \mu\text{g ml}^{-1}$ amphotericin and 20% foetal calf serum (FCS) and plated in 1% gelatine-coated culture dishes. The cells were grown at 37°C in a 5% CO_2 humidified incubator. The following day the medium was changed to remove erythrocytes and was renewed twice a week. After 4–6 days cells formed confluent monolayers and were further subcultured by trypsinisation (0.25%); cell plating efficiency after trypsinization was $>98\%$. The cell population, thus obtained, consisted of $>95\%$ endothelial cells verified by their cobblestone morphology and immunofluorescence staining with antibody against von-Willebrand-factor antigen (Dako, Dakopatts, Denmark). Cells were used in the second and third passage.

Assessment of [^3H]thymidine incorporation in cultured endothelial cells

HUVEC were allowed to grow until confluency. Cells were then washed twice with phosphate buffered saline (PBS) and synchronized by serum starvation for 48 h in medium 199 containing 0.2% bovine serum albumin. Subsequently, the cells were removed from their plastic support by brief trypsinisation, washed and seeded at $1-2 \times 10^4$ cells/well in flat bottomed 96-well tissue culture plates in medium 199 containing 5% FCS. After 2 h, which allowed the cells to adhere, the compounds indicated in the respective figures were added. [^3H]thymidine (specific activity $\sim 2 \text{ Ci mmol}^{-1}$) was added 24 h later to a final concentration of $1 \mu\text{Ci ml}^{-1}$. After an additional 24 h period, cells were lysed by a freeze-thaw cycle and the radioactivity incorporated into DNA was trapped onto glassfibre filters using a Skatron cell harvester. Determinations were carried out in quadruplicate. The amount of [^3H]thymidine incorporated in the presence of 5% FCS (= control) varied between 2,000 and 10,000 c.p.m. in individual experiments with different cell batches. This interassay variance arises in part from modest differences in cell numbers and in the specific activity of [^3H]thymidine; a further factor is the length of transition from G_0/G_1 - to S-phase, which we found to vary between 24 h and 36 h under our assay conditions in individual cell batches. We stress that all effects of the compounds tested are not affected by these variations. In order to allow for interassay comparisons, the amount of [^3H]thymidine incorporated in the presence of 5% FCS was set as 100% and the drug-induced changes were related to this value.

Short-pulse conditions

In experiments in which the time-dependent effect of ^8Br -cyclic AMP was determined (Figure 5b), [^3H]thymidine

($6-8 \mu\text{Ci ml}^{-1}$) was added after 48 h and the incubation was continued for an additional 3 h; thereafter the samples were processed as described above.

Determination of cell number

HUVEC were allowed to grow until confluency, washed twice with PBS and synchronized by serum starvation for 48 h. After trypsinisation cells were seeded at $\sim 1.5 \times 10^5$ cells/well in flat bottomed 12-well tissue culture plates in medium 199 containing 5% FCS. After 2 h the compounds indicated in the legend to Figure 2 were added. Cells were dispersed 72 h thereafter with 0.25% trypsin and counted in a coulter counter (Coulter Electronics).

Determination of intracellular adenosine levels

The intracellular nucleoside- and nucleotide-pool was prelabelled by incubating HUVEC ($\sim 5 \times 10^5$ /well) with [^3H]adenine ($10 \mu\text{Ci/well}$) for 24 h. The [^3H]adenine containing medium was then replaced by medium containing the compounds indicated in Figure 2a. After 6, 12 and 24 h the medium was rapidly removed, the cells were washed in phosphate buffered saline and intracellular nucleosides and nucleotides were extracted with perchloric acid and analysed by h.p.l.c. on a Nucleosil-10- C_{18} reverse-phase column as described (Nees *et al.*, 1985). Chromatographic conditions were as follows: a flow rate of 100 ml h^{-1} , a linear elution gradient of 0.1 M potassium phosphate (pH 5.8) vs. 60% methanol in 0.1 M potassium phosphate (pH 5.8) over 60 min. The fractions corresponding to adenine nucleotides, adenosine, inosine and cyclic AMP were identified from the u.v. signal generated by the addition of unlabelled standards, collected and counted for radioactivity.

Adenylyl cyclase assay

HUVEC membranes were prepared in buffer containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA and $20 \mu\text{M}$ aprotinin. After a rapid freeze-thaw cycle in liquid nitrogen, sucrose was added to a final concentration of 0.25 M; thereafter cells were homogenized by means of an Ultraturrax and centrifuged for 10 min at 20,000 g. The resulting membrane pellet was washed twice in sucrose-free buffer, resuspended at a protein concentration of $\sim 5 \text{ mg ml}^{-1}$, frozen in liquid nitrogen and stored at -80°C . The protein concentration was determined by dye binding with the Bio-Rad Coomassie blue kit using bovine serum albumin as standard. Adenylyl cyclase activity was determined in a final volume of 0.1 ml containing (mM composition): HEPES NaOH 40, (pH 8), EDTA 1, MgCl_2 2, [^{32}P]ATP (specific activity $\sim 200 \text{ cpm pmol}^{-1}$) 0.05, phosphocreatine 5, RO 20-1724 0.1, 5 units of creatinine kinase, 2 units of adenosine deaminase and $10 \mu\text{g}$ of membrane protein; activating ligands were present at the concentrations indicated in Figure 4. After 20 min at 25°C , the reaction was quenched and the [^{32}P]cyclic AMP formed was separated from the substrate by sequential chromatography on Dowex 50W-X8 and neutral alumina (Salomon *et al.*, 1974) with [^3H]cyclic AMP as an internal standard (recovery $\sim 70\%$).

Detection of G-protein α -subunits by immunoblots and Pertussis toxin-catalyzed ADP-ribosylation

For immunoblot analysis, HUVEC membrane samples ($75 \mu\text{g}$ of membrane protein) were resolved by SDS polyacrylamide gel electrophoresis (running gel: 10% acrylamide and 0.13% N,N'-methylene bisacrylamide) and proteins transferred to nitrocellulose. The following antisera were used at a 1:250 dilution: 84 (raised against the peptide KENLKDCGLF), which recognizes all three subtypes of G_i , and CS1 (raised against the peptide RMHLRQYELL), which is specific for $G_{s\alpha}$ (Selzer *et al.*, 1993). The antigen-antibody complexes

were visualized by a second antibody coupled to alkaline phosphatase using the ProtoBlot II kit (Promega). Recombinant G protein α -subunits (G_{ss} , $G_{\text{ss-L}}$, $G_{\text{ir-1}}$, $G_{\text{ir-2}}$) were purified from bacterial lysates as described (Freissmuth & Gilman, 1989; Linder *et al.*, 1990) and used as standards to verify comigration of the bands detected by the antisera. Labelling with pertussis toxin-catalyzed ADP-ribosylation was carried according to Bokoch *et al.* (1983): To reduce background labelling, G proteins were solubilized from the membranes prepared from HUVEC pretreated with increasing concentrations of pertussis toxin (as indicated in the legend to Figure 6). Incubation was 1 h on ice at a protein concentration of 4 mg ml^{-1} in the presence of 25 mM CHAPS; unsolubilized material was removed by centrifugation. The labelling reaction was carried out with an aliquot of the supernatant corresponding to $50 \mu\text{g}$ membrane protein for 40 min in the presence of $[^3\text{O}]\text{-NAD}$; subsequently, the reaction mixture was dissolved in Laemmli sample buffer containing 2% SDS and 40 mM dithiothreitol, and proteins were resolved on a SDS-polyacrylamide gel (running gel: 10% acrylamide and 0.13% N,N' -methylene bisacrylamide). A bovine brain G protein preparation containing a mixture of G_i and G_o was purified according to Sternweis & Robishaw (1984) and used as a standard.

Data are reported as means $\pm$ s.d. from n independent experiments; intra-assay replicate variation was between 5–10% of the mean. All statistical comparisons were made with Student's t test for paired data.

Materials

Cell culture media were from GIBCO (Grand Island, NY), cell culture dishes from Greiner (Krems, Austria). $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$, $[^3\text{H}]\text{-thymidine}$, $[^3\text{H}]\text{-adenine}$ were obtained from NEN. Cholera toxin and $\text{N}^6\text{-cyclopentyladenosine}$ (CPA), 4-nitrobenzylthioinosine (NBMPR), (-)-isoprenaline, 8-bromo cyclic AMP ($^8\text{Br-cyclicAMP}$), collagenase (Type IV) and forskolin were from Sigma (St. Louis, MO, U.S.A.), pertussis toxin from Peninsula Laboratories (St. Helens, UK); GTP γ S, R- and S- N^6 (phenylisopropyl)adenosine (PIA), creatine kinase and adenosine deaminase were from Boehringer Mannheim (Germany); xanthine amine congener (XAC) was from Research Biochemicals (Natick, MA, U.S.A.), 5'-N-ethylcarboxamidoadenosine (NECA), 5'-N-ethylcarboxamido-2-[4-(2-carboxyethyl)phenethyl]adenosine (CGS 21680), DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-20-1724), erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutane-2-ol (ICI 118,551) were generous gifts of Byk Gulden (Konstanz, Germany), Ciba-Geigy (Basel, CH), Hoffman-La Roche (Basel, Switzerland) and ICI (Cheshire, UK), respectively, Sodium-4-[2-(2-hydroxy-(3-chlorophenyl) ethylamino)-propyl] phenoxyacetate (BRL 37344) was kindly supplied by Dr A.D. Strosberg (ICGM, Paris).

Results

Determination of the adenosine receptor subtype

Incubation of HUVEC with adenosine deaminase in the presence of 5% FCS resulted in a significant ($P < 0.001$; $n = 14$) reduction of $[^3\text{H}]\text{-thymidine}$ incorporation (Figure 1a), which varied between 20 and 30% (mean = $27 \pm 2\%$) in different cell batches. This effect of adenosine deaminase was concentration-dependent and abolished by heat-inactivation of the enzyme (data not shown). Similarly, addition of the potent adenosine receptor antagonist, XAC resulted in a concentration-dependent inhibition of $[^3\text{H}]\text{-thymidine}$ incorporation (Figure 1a); the maximal inhibition obtained with XAC was not statistically different from the effect of adenosine deaminase. The half-maximal effect was observed at $\sim 10 \text{ nM}$, a concentration range at which this compound interacts with A_1 and A_2 -adenosine receptors, but does not

cause inhibition of cyclic nucleotide phosphodiesterases (Galen *et al.*, 1992). If the non-selective agonist NECA was added at a concentration of $10 \mu\text{M}$, representing a 100 fold excess of NECA's maximally effective concentration the inhibitory effect of XAC was reversed (Figure 1a, insert; $P < 0.01$, $n = 4$). These findings indicate that endogenous adenosine present in the culture medium contributes to endothelial cell proliferation and imply a receptor-mediated mechanism of action. This assumption was verified by determining the effects of synthetic adenosine receptor agonists on DNA-synthesis in the presence of adenosine deaminase (Figure 1b); the efficacy and potency of CGS 21680 and N-ethylcarboxamidoadenosine (NECA) were found to be comparable with an EC_{50} of about 10 nM , and the effect of

Figure 1 Effect of adenosine deaminase and adenosine receptor ligands on foetal calf serum (FCS)-induced $[^3\text{H}]\text{-thymidine}$ incorporation. (a) Inhibition of $[^3\text{H}]\text{-thymidine}$ incorporation in synchronized HUVEC ($\sim 1.1 \times 10^4$ cells/well) stimulated by 5% FCS (□), by 5% FCS + 2 u ml^{-1} adenosine deaminase (○), 5% FCS + QAC (●). Insert: The effect of XAC (100 nM, open column) is antagonized by addition of NECA ($10 \mu\text{M}$, cross hatched column, $P < 0.01$, $n = 4$). (b) Modulation of $[^3\text{H}]\text{-thymidine}$ incorporation stimulated by 5% FCS (□), FCS in the presence of 2 u ml^{-1} adenosine deaminase alone (○), and combined with CGS 21680 (▲), NECA (●), or CPA (▼). Data represent means from three to four experiments carried out in quadruplicate under the conditions outlined under Methods; $[^3\text{H}]\text{-thymidine}$ incorporation in the presence of 5% FCS was set as 100%.

these agonists was antagonized by XAC (data not shown). The concentration-response curves for cyclopentyl-adenosine (CPA, Figure 1a) and the R- and S-isomers of N⁶-phenylisopropyladenosine (PIA, data not shown) were virtually identical; these compounds were found to be less potent than NECA and CGS 21680, a pharmacological profile consistent with the involvement of an A_{2A}-adenosine receptor. The observed changes in [³H]-thymidine incorporation reflect an increased replication rate of HUVEC which was verified by carrying out a cell count assay over 72 h in the presence of FCS alone (Figure 2b, ▽), addition of adenosine deaminase (Figure 2b, □), and addition of adenosine deaminase + CGS 21680 (Figure 2b, ■). The results were in close agreement to the observations obtained by determining [³H]-thymidine incorporation and also correspond to previously published results (Meininger & Granger, 1990; Ethier *et al.*, 1993).

Apart from interacting with cell-surface receptors, adenosine may exert some of its action via an intracellular site of action, e.g. via interaction with an A₂-like binding site for adenosine and adenosine analogues, in particular NECA (Hutchinson & Fox, 1989), or by binding to the inhibitory P-site of adenylyl cyclase (Olah & Stiles, 1992). Endothelial cells are known to have a very active adenosine metabolism characterized by a large capacity for uptake and release of the nucleoside (Nees *et al.*, 1985). We have tested whether increases in the intracellular level of adenosine were capable of altering the rate of endothelial cell proliferation by employing the adenosine transport inhibitor, NBMPR. Intracellular adenosine levels were determined after 6, 12 and 24 h, a time period relevant for triggering mitogenic activity. Addition of 1 µM NBMPR, a concentration leading to an almost complete inhibition of the nucleoside transporter (data not shown), resulted in a pronounced increase ($P < 0.01$) of intracellular adenosine. The effect of NBMPR at the 12 h time point is shown in Figure 2a (bottom panel); in contrast, the presence of adenosine deaminase had no detectable effect on intracellular adenosine levels (Figure 2a, middle panel). Intracellular adenosine levels expressed as a percentage of the total incorporated radioactivity were $0.28 \pm 0.09\%$, 0.26 ± 0.13 , and 0.84 ± 0.04 for FCS, ADA, and NBMPR, respectively (mean $\pm$ s.d., $n = 3$, $P < 0.01$ for NBMPR vs FCS or ADA). The effect of NBMPR was comparable at the other two time points (i.e. 6 h and 24 h). Intracellular levels of cyclic AMP, which elutes immediately after adenosine, did not reveal any significant changes, irrespective of the time point and of whether NBMPR or adenosine deaminase were present (12 h-values $0.76 \pm 0.10\%$, $0.83 \pm 0.13\%$, and $0.73 \pm 0.24\%$ for FCS, ADA, and NBMPR, respectively, means $\pm$ s.d., $n = 3$). Due to the affinity of NBMPR for the nucleoside transporter this compound also inhibits [³H]-thymidine uptake; a cell count assay was therefore carried out to measure the effect of NBMPR treatment on HUVEC proliferation. As can be seen from Figure 2b, NBMPR did not produce any change in cell number. A cytotoxic effect was ruled out by trypan blue exclusion. Taken together, these findings rule out an intracellular site of action for adenosine and stress the importance of the A_{2A}-receptor as the site of action for the mitogenic activity of adenosine analogues.

Determination of the G protein involved

Since A_{2A}-adenosine receptors are coupled to activation of adenylyl cyclase via G_s, we investigated whether the β₂-adrenoceptor is also capable of mediating endothelial cell proliferation. The β₂-adrenoceptor is a G_s-coupled receptor expressed on endothelial cells (Steinberg *et al.*, 1984; Freissmuth *et al.*, 1986). At low concentrations, isoprenaline (EC₅₀ ~3 nM) stimulates [³H]-thymidine incorporation in the presence of adenosine deaminase (Figure 3a); in the absence of adenosine deaminase it exerts only a minor stimulatory effect. The requirement of adenosine deaminase for the obser-

vation of a stimulatory effect of isoprenaline suggested that the β-adrenoceptor and the A_{2A}-adenosine receptor shared a common signal transduction pathway. A further hint at a common mechanism is that combination of NECA and isoprenaline in maximally active concentrations did not enhance [³H]-thymidine incorporation when compared to the sole addition of each agonist (Figure 3b). In order to define the

Figure 2 (a) H.p.l.c. analysis of the intracellular adenine nucleotide and nucleoside pool following treatment with adenosine deaminase (ADA, middle) and 4-nitrobenzylthioinosine (NBMPR, bottom): 5×10^5 cells/well were prelabelled with [³H]-adenine for 24 h and subsequently incubated in the presence of FCS (top), $2 \mu\text{M}$ adenosine deaminase (ADA, middle) or $1 \mu\text{M}$ NBMPR (bottom) for 12 h. A neutralized perchloric acid extract was analysed by h.p.l.c. as described under Methods. The region of the chromatogram is shown, in which adenosine and cyclic AMP elute; no differences were noted for adenine nucleotides and the breakdown product inosine, which elute earlier. (b) Effect of NBMPR, CGS 21680 and pertussis toxin on cell number. For the determination of cell number, synchronized cells were plated at 1.5×10^5 cells/well in 5% FCS (FCS, ▽) and the combination of FCS and the following compounds: NBMPR (▲), 10 ng mg^{-1} pertussis toxin (PTX, ▼; $P < 0.05$ vs. FCS), $2 \mu\text{M}$ adenosine deaminase (ADA, □; $P < 0.05$ vs. FCS), and adenosine deaminase + $1 \mu\text{M}$ CGS 21680 (CGS, ■; $P < 0.05$ vs. ADA). Cell number was determined 72 h after plating; the value determined in the presence of 5% FCS ($2.2 \pm 0.4 \times 10^5$ cells) was set as 100%.

pharmacological subtype of the β -adrenoceptor we used the β_3 -selective agonist, BRL 37344 (Emorine *et al.*, 1989) and the highly β_2 -selective antagonist ICI 118,551. BRL 37344 did not exert any effect ruling out the involvement of a β_3 -adrenoceptor. On the other hand, the stimulatory effect of isoprenaline was competitively antagonized by ICI 118,551 (Figure 3a). A pA_2 of ~ 9 was estimated from the ICI 118,551-induced shift of the concentration-response curve for isoprenaline; this is in agreement with a K_D of ~ 1 nM determined for the interaction of ICI 118,551 with endothelial β_2 -adrenoceptors (Freissmuth *et al.*, 1986). These findings establish that stimulation of the β_2 -adrenoceptor generates a mitogenic signal in human endothelial cells.

Figure 3 Effect of β -adrenoceptor ligands on foetal calf serum (FCS)-induced $[^3H]$ -thymidine incorporation. (a) $[^3H]$ -thymidine incorporation was determined in synchronized HUVEC ($\sim 1.2 \times 10^4$ cells/well) prestimulated by 5% FCS (O), by 5% FCS + 2 μ l ml^{-1} adenosine deaminase (●), 5% FCS + isoprenaline in the presence of 2 μ l ml^{-1} adenosine deaminase (□), isoprenaline in the presence of adenosine deaminase and 10 nM of the β_2 -selective antagonist, ICI 118,551 (■), and 5% FCS + the β_3 -selective agonist BRL 37,344 in the presence of adenosine deaminase (▼). (b) $[^3H]$ -thymidine incorporation in synchronized HUVEC in the presence of 2 μ l ml^{-1} adenosine deaminase (ADA), the combination of adenosine deaminase and 1 μ M NECA (+NECA), adenosine deaminase and 100 nM isoprenaline (+Iso), adenosine deaminase and 1 μ M NECA and 100 nM isoprenaline (+NECA + Iso). Data represent means from three to five experiments carried out in quadruplicate; $[^3H]$ -thymidine incorporation in the presence of 5% FCS was set as 100%.

The coupling of A_2 -adenosine receptors to activation of adenylyl cyclase has been described on microvascular and endothelial cell membrane preparations obtained from different species and vascular beds (Schütz *et al.*, 1982; Des Rosiers & Nees, 1987). Also in HUVEC, CGS 21680, NECA and isoprenaline were found to stimulate adenylyl cyclase activity with comparable efficacies (Figure 4); EC_{50} -values were 0.3 ± 0.2 , 0.52 ± 0.08 and 0.09 ± 0.03 μ M (mean $\pm$ s.d.; $n = 3$) for CGS 21680, NECA and isoprenaline, respectively. In contrast, the A_1 -selective agonist, CPA (not shown) and the β_3 -agonist BRL 37,344 (Figure 4b) failed to exert any effect up to concentrations of 10 μ M.

Since the endothelial A_{2A} -adenosine receptor and the β_2 -adrenoceptor were coupled to both adenylyl cyclase activation and stimulation of $[^3H]$ -thymidine incorporation, we investigated whether a rise in intracellular cyclic AMP-levels

Figure 4 Effect of adenosine and β -adrenoceptor ligands on adenylyl cyclase activity in HUVEC membranes. (a) Membranes (10 μ g of protein) prepared from HUVEC were incubated for 20 min in the presence of 10 nM GTP γ S + CGS 21680 (●) and GTP γ S + NECA (▲). (b) Membranes (10 μ g of protein) prepared from HUVEC were incubated for 20 min in the presence of 10 nM GTP γ S + isoprenaline (■), and GTP γ S + BRL 37344 (□). Basal activity, i.e. activity in the presence of GTP γ S alone was 30 ± 3 pmol cyclic AMP min^{-1} mg^{-1} and has been subtracted. Data are from a representative experiment carried out in triplicate; 2 additional experiments gave similar results.

directly mediates cell proliferation; agents known to raise intracellular cyclic AMP levels (i.e. forskolin) or to activate protein kinase A directly (i.e. ^8Br -cyclic AMP) were used. Rather surprisingly, both compounds exerted a concentration-dependent inhibition of [^3H]-thymidine incorporation (half-maximum inhibition occurring at 0.3 ± 0.1 and $14 \pm 5 \mu\text{M}$ forskolin and ^8Br -cyclic AMP, $n = 3$, respectively), the maximum effect being comparable (Figure 5a). These effects were independent of the presence of adenosine deaminase (data not shown).

G proteins can control more than one effector system e.g. G_s is known to stimulate adenylyl cyclase as well as opening of voltage-sensitive calcium channels (Spiegel *et al.*, 1992). We therefore tested whether direct activation of G_s by cholera toxin, which constitutively activates G_s , can mimic the action of adenosine analogues. However, addition of cholera toxin (Figure 5a, hatched columns) decreased [^3H]-thymidine incorporation to a similar extent as forskolin and ^8Br -cyclic AMP. The concentration of cholera toxin employed (10 ng ml^{-1}) was maximally active; no additional inhibition was obtained, if the concentration of the toxin was raised to 100 ng ml^{-1} . These data obtained with cholera toxin argue against an involvement of G_s .

Due to desensitization, receptor-mediated increases of cyclic AMP are generally characterized by rapid fading in spite of continuous presence of agonists. We therefore designed an additional experiment to test the hypothesis that short-term increases in cyclic AMP are responsible for the A_{2A} -adenosine and β_2 -adrenoceptor-induced increase in DNA-synthesis. ^8Br -cyclic AMP was added to the incubation medium for varying time periods in the presence and absence of adenosine deaminase (Figure 5b). Short-term incubation (1–5 h) failed to produce a stimulatory effect and to reverse the inhibitory action of adenosine deaminase; this effect would have been expected if the mitogenic effect of adenosine were mediated via a transient rise of cyclic AMP. Furthermore, at longer incubation times ($> 16 \text{ h}$), ^8Br -cyclic AMP causes a progressive inhibition of DNA-synthesis independent of whether adenosine deaminase is present or not in the culture medium. Both observations indicate distinct intracellular pathways for the inhibitory effect of cyclic AMP and the inhibitory effect of adenosine deaminase (and therefore the stimulatory effect exerted by the A_{2A} -adenosine and the β_2 -adrenoceptor). The inhibitory effect of ^8Br -cyclic AMP after a 48 h incubation period in Figure 5b seems more pronounced than that given in Figure 5a; however, this discrepancy arises from the different assay conditions. Since in the experiments depicted in Figure 5b [^3H]-thymidine was present for the last 3 h of the incubation, only those cells that are in S-phase during this short period, incorporate the label. In all other experiments [^3H]-thymidine was present for 24 h, i.e. from the time point when cells enter S-phase, and thus an integrative response is obtained.

Under appropriate conditions, the mammalian β_2 -adrenoceptor, albeit a prototypical G_s -coupled receptor, can be shown to interact with G_i (Abramson & Molinoff, 1985; Asano *et al.*, 1984; Freissmuth *et al.*, 1991); we therefore tested whether the mitogenic response promoted by activation of the A_{2A} -adenosine receptor was sensitive to pertussis toxin, which disrupts receptor-G protein coupling by ADP-ribosylating a cysteine-residue in the carboxy terminus of the α -subunits of the G_i - and G_o -group. Addition of pertussis toxin *per se* resulted in a concentration-dependent decrease of [^3H]-thymidine incorporation and cell number, respectively, with half-maximal effect of about 1 ng ml^{-1} (Figures 6a and 2b). The antiproliferative effect of pertussis toxin correlated with a loss of ADP-ribosylation substrate in membranes subsequently prepared from pertussis toxin-treated cells (Figure 6b). Under electrophoretic conditions, which allow resolution of several pertussis toxin-substrates in a bovine brain G protein preparation, a single labelled band is seen in membrane extracts from HUVEC. We identified this band by immunoblots as $G_{i\alpha-2}$ based on comigration with the $rG_{i\alpha-2}$ -

standard (Figure 6c); neither $G_{i\alpha-1}$ nor $G_{i\alpha-3}$, which comigrate under our electrophoretic conditions but can be resolved from $G_{i\alpha-2}$ (Selzer *et al.*, 1993), were detected in 3 different

Figure 5 Modulation of foetal calf serum (FCS)-induced [^3H]-thymidine incorporation by compounds targeting the cyclic AMP-signaling cascade. (a) [^3H]-thymidine incorporation in synchronized HUVEC ($\sim 1.1 \times 10^4$ cells/well) was stimulated by 5% FCS and the indicated combinations of 5% FCS + compound; [^3H]-thymidine incorporation in the presence of FCS alone varied between 2,000 and 10,000 c.p.m. in individual experiments and was set at 100% for the purpose of normalisation. ADA: $2 \mu\text{M}$ adenosine deaminase; + CTX10: 10 ng ml^{-1} cholera toxin combined with $2 \mu\text{M}$ ADA; + FSK10: $10 \mu\text{M}$ forskolin combined with $2 \mu\text{M}$ ADA; + ^8Br -cyclic AMP: 1 mM ^8Br -cyclic AMP combined with $2 \mu\text{M}$ ADA. Data are means $\pm$ s.e. mean from 3 to 6 experiments carried out in quadruplicate under the conditions outlined under Methods. All reductions in [^3H]-thymidine incorporation are significant ($P < 0.01$) when compared to the paired FCS-control (*t*-test for paired data). (b) [^3H]-thymidine incorporation in synchronized HUVEC ($\sim 1.1 \times 10^4$ cells/well) was stimulated by 5% FCS (A) 5% FCS + $2 \mu\text{M}$ adenosine deaminase (C); 5% FCS + 1 mM ^8Br -cyclic AMP (B) and 5% FCS + $2 \mu\text{M}$ adenosine deaminase + 1 mM ^8Br -cyclic AMP (D) for 1, 2, 5, 16 and 48 h. After this time, medium was replaced by 5% FCS or 5% FCS + $2 \mu\text{M}$ adenosine deaminase. Using short-pulse conditions as outlined under Methods, [^3H]-thymidine was added for only 3 h. Statistical analysis revealed $P < 0.01$ for adenosine deaminase vs 5% FCS at all time points as well as $P < 0.01$ for ^8Br -cyclic AMP vs 5% FCS and ^8Br -cyclic AMP + ADA vs ADA after 16 and 48 h of incubation.

Figure 6 (a) Inhibition of serum-induced $[^3\text{H}]$ -thymidine incorporation in HUVEC by pertussis toxin. About 10^6 cells were synchronized by serum starvation for 48 h and incubated with 5% FCS in the absence and presence of increasing concentrations of pertussis toxin; $[^3\text{H}]$ -thymidine incorporation was assayed using 1×10^4 cells/well. Data represent means from four experiments carried out in quadruplicate; $[^3\text{H}]$ -thymidine incorporation in the presence of 5% FCS was set as 100%. (b) Pertussis toxin-catalyzed ADP-ribosylation: the remainder of the cells were maintained in the absence of $[^3\text{H}]$ -thymidine and harvested after 24 h. A membrane extract was prepared from these cells and subjected to pertussis-toxin catalyzed ADP-ribosylation by using $[^3\text{P}]\text{-NAD}$ and resolved by SDS polyacrylamide gel electrophoresis. Lane St: purified bovine brain G_i/G_o ; lane C: control cells maintained in the absence of pertussis toxin; lanes 0.1, 1, 10, 100, cells pretreated with the respective concentrations of pertussis toxin. (c) Immunoblot analysis of $G_{i\alpha}$ and $G_{s\alpha}$ in HUVEC membranes: membranes (75 μg) of 3 different batches of HUVEC (lanes CDE) were resolved on an SDS polyacrylamide gel and probed with antiserum 84 (specific for the three subtypes of $G_{i\alpha}$) and CS1 (specific for $G_{s\alpha}$); lane A: 10 ng r $G_{i\alpha 2}$, lane B: 100 ng r $G_{i\alpha 1}$, lane F: 50 ng r $G_{s\alpha-L}$, lane G: 50 ng r $G_{s\alpha-S}$. Only the 35 to 55 kDa region of the gel is shown.

HUVEC preparations. Both the short and the long splice variant of $G_{i\alpha}$, $G_{i\alpha-S}$, $G_{i\alpha-L}$, were detected (Figure 6c). These experiments indicate an important role of G_{i2} in mediating serum-stimulated growth of HUVEC. However, pertussis toxin, which itself causes a profound inhibition of cell growth, abolished neither the stimulatory effect of CGS 21680 nor the inhibitory effect of adenosine deaminase (Figure 7).

Discussion

Four types of G protein-coupled adenosine receptors have been identified by molecular cloning, namely A_1 , A_{2A} and A_{2B} and A_3 -receptor (Maenhaut *et al.*, 1990; Townsendsnicholson & Shine, 1992; Pierce *et al.*, 1992; Zhou *et al.*, 1992), which can also be differentiated by their pharmacological profile. In the present paper, we show that the proliferation of HUVEC

Figure 7 Influence of pretreatment of HUVEC with pertussis toxin on A_2 -receptor mediated stimulation of $[^3\text{H}]$ -thymidine incorporation. Synchronized HUVEC were plated at a density of 1×10^4 cells/well in 5% FCS followed by the addition of 10 ng ml^{-1} pertussis toxin (open column) or the combination of 10 ng ml^{-1} pertussis toxin + 2 u ml^{-1} adenosine deaminase (hatched and cross-hatched columns); after 6 h, $1 \mu\text{M}$ NECA (cross hatched column) or vehicle (open and hatched columns) was added. Data shown are means $\pm$ s.d. from 4 separate experiments, $[^3\text{H}]$ -thymidine incorporation in the presence of 10 ng ml^{-1} PTX was set as 100%; * $P < 0.01$.

is stimulated by adenosine analogues via activation of a receptor that meets the criteria of the A_{2A} -subtype, namely high-affinity for NECA and CGS 21680, low affinity for N^6 -substituted analogues such as CPA and potent antagonism by the methylxanthine XAC (Galen *et al.*, 1992). Mediation via an A_1 -adenosine receptor, which displays stereoselectivity for the *R*-isomer of PIA and high affinity for CPA, is unequivocally ruled out (Galen *et al.*, 1992). Antagonism of the mitogenic activity by the methylxanthine XAC excludes an A_3 -adenosine receptor. This subtype has recently been described and its prominent pharmacological feature is the lacking sensitivity to the antagonistic effect of methylxanthines; in addition, the affinity of CGS 21680 for the A_3 -adenosine receptor is one order of magnitude lower than that of NECA (Zhou *et al.*, 1992). Similarly, CGS 21680 interacts only weakly with the A_{2B} -adenosine receptor (Pierce *et al.*, 1992).

Previous work has established that adenosine stimulates proliferation of endothelial cells, but did not provide information on the receptor-subtype involved. Our findings are in contrast to the conclusion reached by Van Daele *et al.* (1992), who reports that only the adenosine precursor ATP, ATP-analogues (P_2 -receptor agonists) and adenosine itself initiate DNA-synthesis in bovine endothelial cells, whereas typical adenosine receptor agonists and antagonists were without effect. We do not believe that this discrepancy arises from the different origin of the endothelial cells, both with respect to species and to the vascular bed; instead, the differing results are most probably attributable to the variation in assay conditions, in particular the absence of foetal calf serum and therefore of serum-derived growth factors in the study of Van Daele *et al.*; in serum-free medium A_{2A} -adenosine as well as β_2 -adrenoceptor agonists failed to stimulate $[^3\text{H}]$ -thymidine incorporation, whereas the phorbol ester phorbol 12-myristate 13-acetate (TPA, an activator of the protein kinase C pathway) was still capable of inducing a profound increase in DNA synthesis (data not shown). This interpretation is also consistent with the concept that growth

stimulation via most G protein-coupled receptors requires the concomitant presence of an additional stimulus, typically activation of growth factor-sensitive tyrosine kinase-receptor linked pathways that generate obligatory signals for the progression through the cell cycle and entry into S-phase (Rozenfurt, 1986; Pouyssegur & Seuwen, 1992). The tentative intracellular site of action for adenosine described by Van Daele *et al.* (1992) remains enigmatic, but our experiments, in which elevation of the intracellular adenosine concentration by the adenosine transport inhibitor, NBMPR, failed to change the rate of endothelial cell proliferation strongly argue against the involvement of an intracellular site of action.

It has long been known that β_2 -adrenoceptors are present on endothelial cells, although their functional significance remained elusive. Recent work has demonstrated that trans-endothelial exchange of macromolecules is inhibited by activation of β_2 -adrenoceptors, an effect mediated by cyclic AMP (Zink *et al.*, 1993). In the present work, we show that activation of endothelial β_2 -adrenoceptors also produces a long-term effect, namely stimulation of endothelial cell growth, and that this effect is independent of increases in intracellular cyclic AMP (see below). Given the fact that endothelial cells can be exposed to high levels of catecholamines *in vivo*, one is led to suspect that circulating adrenaline and/or locally released noradrenaline act in concert with other angiogenic factors to promote endothelial cell growth.

The effector system that is believed to mediate most if not all biological effects of the A_2 -adenosine receptor (and of the β_2 -adrenoceptor) is adenylyl cyclase (Galen *et al.*, 1992; Stiles, 1992; Collis & Hourani, 1993). Stimulation of adenylyl cyclase consistent with the pharmacological profile of an A_{2A} -receptor was also observed with the non-selective agonist NECA and the A_{2A} -selective agonist CGS 21680 in HUVEC membranes, but their potencies are clearly lower when compared to their potencies to enhance DNA-synthesis; a similar observation was also made for isoprenaline (EC_{50} 3 nM for proliferation, 90 nM for stimulation of adenylyl cyclase). This discrepancy between EC_{50} -values obtained in broken cell preparation and intact cells has been ascribed to the fragmentation of the membrane which results in limited receptor mobility, thereby reducing the signal amplification built into the catalytic nature of the receptor-G protein interaction (Stickle & Barber, 1989). Hence, we do not interpret the observed difference in potency for adenosine analogues and isoprenaline as an indication that different receptor subtypes mediate stimulation of endothelial adenylyl cyclase activity and of cell proliferation. However, this possibility cannot be ruled out.

Irrespective of whether the same A_{2A} -receptor or two different receptors with a related pharmacological profile are linked to an increase in intracellular cyclic AMP levels and to endothelial cell proliferation, this effector system is not responsible for the mitogenic effect of A_{2A} -adenosine and β -adrenoceptor agonists. All compounds that activate the cyclic AMP-cascade downstream of the receptor, i.e. at the level of G_s , adenylyl cyclase or protein kinase A, decreased DNA-synthesis. The role of cyclic AMP in promoting cell growth is cell type-dependent: in several specialized cells, such as brown fat, Schwann cells as well as endocrine cells elevated cyclic AMP levels exert a stimulatory effect (Dumont *et al.*, 1989; Morgan *et al.*, 1991; Bronnikov *et al.*, 1992). In others such as lymphocytes and smooth muscle cells, a sustained

increase in cyclic AMP is associated with inhibition of proliferation (Souness *et al.*, 1992; Skálhegg *et al.*, 1992). Our observations in HUVEC are consistent with those obtained in endothelial cells derived from several species as well as from different vascular beds (Leitman *et al.*, 1986; Moodie *et al.*, 1991), where cyclic AMP-analogues, phosphodiesterase-inhibitors and forskolin reduced proliferation. All these manipulations are predicted to result in long-lasting increases in intracellular cyclic AMP. Based on brief incubations of bovine endothelial cells with cholera toxin, which caused a stimulation of cell growth, Meininger *et al.* (1990) proposed that the mitogenic effect of adenosine agonists is due to a transient rise of intracellular cyclic AMP. However, this argument appears weak, since (i) cholera toxin acts enzymatically once its A-subunit has penetrated the cell and should thus produce a long lasting rise in cyclic AMP; (ii) the B-subunit of cholera toxin *per se* can act as a mitogen (Spiegel, 1989). We have therefore analysed the effect of varying incubation periods with 3H -cyclic AMP, which failed to stimulate DNA synthesis and to antagonize the inhibitory effect of adenosine deaminase. Furthermore, the inhibitory effect of cyclic AMP on 3H -thymidine incorporation was independent of adenosine deaminase, indicating two distinct pathways. Therefore cyclic AMP does not qualify as the intracellular signal responsible for the mitogenic action of the A_{2A} -adenosine and β_2 -adrenoceptor.

G protein-coupled receptors are promiscuous in that a defined molecular species of receptor can be shown to couple to several types of G proteins that do not necessarily belong to the same functional class (Milligan, 1993). The fact that cholera toxin does not mimic the effect of A_{2A} -adenosine or β_2 -adrenoceptor activation implies a G_s -independent pathway. The involvement of pertussis toxin-sensitive G proteins, in particular the subtypes of G_i , in stimulating cell growth is well documented (Spiegel *et al.*, 1992; Seuwen & Pouyssegur, 1992). Our data suggest an important role of a G_{12} -linked pathway for serum-induced growth of HUVEC and recent work on an endothelial-derived cell line (EA hy926) show that G_i also participates in later steps of angiogenesis (Bauer *et al.*, 1992). However, inactivation of G_{12} by pertussis toxin does not abolish the effect of A_{2A} -adenosine receptor activation on DNA-synthesis.

Recently, a series of experiments had established that the β_2 -adrenoceptor as well as other G_i protein coupled receptors stimulate the Na-H antiporter (Ganz *et al.*, 1990; Barber & Ganz, 1992). This stimulation occurs independent from G_s , but is mediated by a member of the pertussis and cholera toxin-insensitive G_{12} protein subgroup, i.e. G_{13} (Voyno-Yasenetskaya *et al.*, 1994). In addition constitutive activation of G_{12} promotes cellular growth and confers a transformed phenotype (Xu *et al.*, 1993). It is attractive to speculate that the interaction between a member of this G protein subgroup and the A_{2A} adenosine and the β_2 -adrenoceptor is responsible for the proliferative response in HUVECs.

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