Regulation of proliferation of LLC-MK₂ cells by nucleosides and nucleotides: the role of ecto-enzymes

Raf LEMMENS*, Luc VANDUFFEL, Henri TEUCHY and Ognjen CULIC

Limburgs Universitair Centrum, Department MBW, Biochemistry, Universitaire Campus, B-3590 Diepenbeek, Belgium

1. Using the incorporation of [*methyl*-³H]thymidine as a proliferation marker, the effects of various nucleosides and nucleotides on endothelial LLC-MK₂ cells were studied. We found that ATP, ADP, AMP and adenosine in concentrations of 10 μ M or higher stimulate the proliferation of these cells. 2. Inhibition of ecto-ATPase (EC 3.6.1.15), 5'-nucleotidase (EC 3.1.3.5) or alkaline phosphatase (EC 3.1.3.1) significantly diminished the stimulatory effect of ATP, indicating that the effect is primarily caused by adenosine and not by adenine nucleotides. Also, the effect depends only on extracellular nucleosides, since inhibition of nucleoside uptake by dipyrindamole has no influence on proliferation. 3. Other purine nucleotides and nucleosides (ITP, GTP, inosine and guanosine) also stimulate cell proliferation, while pyrimidine nucleotides and nucleosides (CTP, UTP,

cytidine and uridine) inhibit proliferation. Furthermore, the simultaneous presence of adenosine and any of the other purine nucleosides is not entirely additive in its effect on cell proliferation. At the same time any pyrimidine nucleoside, when added together with adenosine, has the same inhibitory effect as the pyrimidine nucleoside alone. 4. Apparently these proliferative effects are neither caused by any pharmacologically known P₁-purinoceptor, nor are they mediated by cyclic AMP, cyclic GMP, or D-*myo*-inositol 1,4,5-trisphosphate as second messenger, nor by extracellular Ca²⁺. 5. Therefore, we conclude that various purine and pyrimidine nucleosides can influence the proliferation of LLC-MK₂ cells by acting on putative purinergic and pyrimidinergic receptors not previously described.

INTRODUCTION

Biological responses to extracellular ATP [1] and adenosine [2] have been documented in virtually every major organ and/or tissue system studied so far. There is a growing body of evidence on the influence of extracellular adenosine and ATP on cell proliferation. This effect is usually described as a phenomenon mediated via P₁- or P₂-purinoceptors on the cell surface [3].

Evidence has been presented that extracellular ATP can stimulate mitogenesis in mammalian cells by different signalling pathways [4–6]. ATP stimulates the proliferation of endothelial [7], smooth muscle [8–10], fibroblast 3T3 and 3T6, and tumour A431 cells [11], glomerular mesangial cells [6,12] and macrophages [13]. In addition, various dinucleotide phosphates can stimulate cell proliferation when present in the extracellular medium [14,15].

Adenosine has been shown to stimulate the proliferation of endothelial [7,16–18], neuroblastoma [19] and mouse fibroblast 3T3 cells [20], but also to inhibit the proliferation of fibroblasts [21] and pericytes [22]. Interesting information about the potential proliferative effect of extracellular adenosine came from a study on thyroid cells in which cDNA for an A₂-receptor was expressed, causing stimulation of cell proliferation even in the absence of externally added adenosine [23]. This finding indicates that adenosine is normally released from the cells, either as such or in the form of ATP and other adenine nucleotides as its precursors [24].

Nucleotides present in all cells can be released through different mechanisms: exocytosis [25,26], transport via intrinsic plasma membrane proteins such as P-glycoprotein [27] and the cystic fibrosis transmembrane conductance regulator [28,29], or cellular

lysis. Mechanical forces applied on cells, like shear stress for example, are also shown to cause release of intracellular ATP [30]. Cells are able to release ATP in response to various agonists, including ATP itself [24]. Therefore, the concept of nucleotides as non-specific (universal) autocrine regulators of cell proliferation attracts a growing attention.

The extracellular metabolism of ATP and other nucleoside phosphates is mainly controlled by enzymes like ecto-ATPase, 5'-nucleotidase and alkaline phosphatase, which are believed to be ubiquitously present on the surface of many different cell types [25]. These enzymes are capable of mediating a cascade dephosphorylation of nucleoside phosphates, generating nucleosides in the extracellular space [31–33]. Since the cellular effects of adenosine and ATP are often very different, with only one of these substances being biologically active, this nucleotide to nucleoside conversion may be an important step in the regulation of cell proliferation.

In this paper we describe the proliferative effects of adenosine and ATP on five randomly chosen cell types. In particular, a more detailed study was made of the effect of various naturally occurring nucleotides and nucleosides on the proliferation of LLC-MK₂ cells. The role of different ecto-nucleotidase enzymes in the regulation of this process was also investigated.

EXPERIMENTAL

Materials

Reactive Blue 2 (RB-2), 8-bromo-cyclic AMP (sodium salt), 8-bromo-cyclic GMP (sodium salt), receptor agonists and

Abbreviations used: IP₃, D-*myo*-inositol 1,4,5-trisphosphate; RB-2, Reactive Blue 2; RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione; DMEM, Dulbecco's modified Eagle's medium; NBCS, newborn-calf serum; AP₄A, P¹,P⁴-di(adenosine-5') tetraphosphate; AP₅A, P¹,P⁵-di(adenosine-5') pentaphosphate.

* To whom correspondence should be addressed.

antagonists, and toxins were purchased from RBI (Natick, MA, U.S.A.). All nucleosides and nucleotides, 5-(*N,N*-dimethyl)-amiloride (hydrochloride), dipyrindamole, diadenosine polyphosphates, adenosine 5'-[α,β -methylene]-diphosphate and forskolin were obtained from Sigma (St. Louis, MO, U.S.A.). Oligomycin, levamisole, EGTA and ouabain (octahydrate) were purchased from Janssen Chimica (Beerse, Belgium). Cerium chloride was from Merck (Darmstadt, Germany). Glutaraldehyde was obtained from Fluka Chemie AG (Buchs, Switzerland), and staurosporine from Calbiochem (CA, U.S.A.). Cell culture products and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione (RO 20-1724) were bought from Life Technologies Inc. (New York, NY, U.S.A.). Radioactive substances came from DuPont de Nemours (Brussels, Belgium). Suramin was kindly provided by Dr. J. A. Boutin (Institut de Recherches Servier, Suresnes, France).

Cell culture

LLC-MK₂ (monkey kidney), HeLa S3 (human carcinoma), Cos-7 (African green monkey kidney, fibroblast-like) and 293 (transformed human kidney, embryonic) cell lines were obtained from ATCC (Rockville, MD, U.S.A.). GM00637F cells (simian virus 40-transformed human skin fibroblasts) were purchased from NIGMS (Camden, NY, U.S.A.). All cell cultures were tested for mycoplasma using the Gibco Mycotect[®] kit (Life Technologies Inc., New York, NY, U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), with penicillin-streptomycin, L-glutamate, sodium pyruvate, non-essential amino acids and 10% (v/v) newborn-calf serum (NBCS). The cells were trypsinized and plated on 96-well plates at a density of 2×10^4 cells per well. After 16 h of incubation, the cells were rinsed once with PBS, and once with serum-free medium, after which the medium was replaced by DMEM without NBCS. After another 48 h of incubation to induce starvation, proliferative agents, inhibitors, agonists or antagonists were added.

Determination of cell number

The cells were left to grow for 48–72 h after addition of the different nucleotides and nucleosides. To determine the cell number, Alamar Blue (BioSource International, Camarillo, CA, U.S.A.) was used. This reagent is an indicator dye, formulated to measure quantitatively the proliferation of cells. It consists of an oxidation–reduction indicator that yields a colorimetric change in response to metabolic activity. The measurements were performed as described in the general procedures provided by the manufacturer.

[methyl-³H]Thymidine incorporation

Together with the different agents, [methyl-³H]thymidine (0.5 μ Ci per well) was added. After 16 h of incubation, the medium was removed and the cells were trypsinized. Using an automated 96-well harvester, the cells were transported to a printed filtermat and their radioactivity was determined by counting in a β -scintillation counter. Except when stated otherwise, all results are expressed as percentage stimulation: (incorporated radioactivity with stimulus/incorporated radioactivity without stimulus $- 1$) $\times 100$.

Cytochemical determination of adenosine production

The cells were trypsinized, seeded on chamber slides (Miles Scientific, Naperville, IL, U.S.A.) and grown for at least 18 h before being washed with Tris-buffered saline (TBS). Fixing

solution contained 2% (w/v) glutaraldehyde, 5% (w/v) sucrose and 0.1 M cacodylate buffer (pH 7.4). Fixation time was 30–60 min. Subsequently, the cells were washed for 2–3 h with 0.1 M cacodylate buffer (pH 7.4) containing 5% (w/v) sucrose and used for cytochemical procedures. The cytochemical detection of cells' capability to produce adenosine from ATP as an initial substrate was carried out in a 30 mM Tris/HCl buffer (pH 7.4), containing 100 mM NaCl, 5 mM KCl, 1 mM ouabain, 5 μ g/ml oligomycin and 4 mM CaCl₂. Additionally, the medium also contained 0.2 mM CeCl₃ as a phosphate-capturing agent and 40 nM [α -³²P]ATP (specific radioactivity was 800 Ci/mmol). After incubation at 37 °C for 90 min, the medium was removed, the upper parts of the chamber slides were detached and the slides were washed three times for 5 min with TBS. The slides were dried at room temperature and dipped in NTB2 autoradiographic emulsion (Eastman Chemical Company, Rochester, NY, U.S.A.) diluted with distilled water in a 1:1 (v/v) ratio. After exposure (typically for 2–3 days) at 4 °C in boxes containing desiccant the slides were developed and inspected under the light microscope.

Diadenosine polyphosphate breakdown

*P*¹,*P*⁴-di(adenosine-5') tetraphosphate (AP₄A) and *P*¹,*P*⁵-di(adenosine-5') pentaphosphate (AP₅A) breakdown was carried out in the same buffer as described for the cytochemical determination of adenosine production. Substrate was 1 mM AP₄A or AP₅A. ATP (1 mM) was used as a positive control. Typically, 10⁶ cells were incubated for 1 h at 37 °C, the reaction was stopped with a 1% SDS solution and the phosphate released was measured by the method of LeBel et al. [34], in which the cells are lysed and the complete phosphate content in the reaction mixture was photometrically determined. This method gives reliable results up to phosphate concentrations of 20 μ M.

Other determinations

For determination of the intracellular cyclic AMP and D-*myo*-inositol 1,4,5-trisphosphate (IP₃) concentrations, the cells were grown as described in the Cell culture section, using 24-well plates at a density of 5×10^4 cells per well. Before the different agents were added, the cells were preincubated with RO 20-1724, an inhibitor of cyclic AMP phosphodiesterase, for at least 1 h. After 1, 3, 5 or 10 min of incubation with different agents, the intracellular cyclic AMP was determined using the cyclic AMP [¹²⁵I] scintillation proximity assay (SPA) system (Amersham International plc, Amersham, Bucks., U.K.).

To determine the intracellular amount of IP₃, the cells were preincubated with 10 mM LiCl for 1 h, and left to incubate with the different agents for 30 s to 10 min, after which IP₃ was determined, using the (IP₃) [³H] assay system from Amersham International plc, U.K.

In order to measure the protein content in the different wells, the cells were first precipitated using ice-cold 10% (w/v) trichloroacetic acid for 20 min, and then solubilized with 0.2 M NaOH, 1% (w/v) SDS at 95 °C for 15 min. A sample was taken to determine the protein content with the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.).

Statistical calculations

Results were tested for significance using the two-tailed Student's *t*-test.

RESULTS

First, we tested the effect of ATP and adenosine on five randomly chosen cell lines: LLC-MK₂, HeLa S3, Cos-7, 293 and GM00637F (Figure 1). LLC-MK₂, Cos-7 and GM00637F cells showed a significant proliferative response to ATP and adenosine. 293 cells were significantly influenced only when ATP or adenosine at a concentration of 100 μ M was applied, while neither of these substrates significantly influenced proliferation of HeLa S3 cells. For a more detailed study of the influence of the nucleosides and nucleotides on cell proliferation we used the LLC-MK₂ cells.

As presented in Figure 2, adenosine, AMP, ADP and ATP significantly stimulate cell proliferation of LLC-MK₂ cells in a concentration-dependent manner. The presence of 10% (v/v) NBS stimulates cell proliferation to a lesser extent than 10 μ M of the adenine nucleotides or adenosine ($21 \pm 3\%$).

We were able to demonstrate adenosine production from ATP as initial substrate, using a cytochemical approach based on [α -³²P]ATP for labelling and Ce³⁺ ions as a phosphate-capturing

Figure 1 Effect of ATP and adenosine on the [$\text{methyl-}^3\text{H}$]thymidine incorporation in different cell lines

All cell lines were brought to starvation for 48 h in serum-free DMEM without NBS, after which ATP (100 μ M, open bars; 10 μ M, closed bars) or adenosine (100 μ M, hatched bars; 10 μ M, cross-hatched bars) was added, together with [$\text{methyl-}^3\text{H}$]thymidine. After 24 h the incorporated radioactivity was determined. Results are mean values ($\pm$ S.D.) for six independent measurements from one typical experiment (* $P < 0.005$; ** $P < 0.05$).

Figure 2 Influence of adenine nucleotides and adenosine on the [$\text{methyl-}^3\text{H}$]thymidine incorporation in LLC-MK₂ cells

Quiescence was induced by incubating the cells for 2 days in serum-free medium. Then [$\text{methyl-}^3\text{H}$]thymidine and the stimulatory agents (ATP, $\circ$; ADP, $\square$; AMP, $\triangle$; adenosine, $\bullet$) were added in the appropriate concentrations. After 24 h, incorporated radioactivity was determined by liquid scintillation counting. Presented data are mean values for six independent measurements from one typical experiment; standard deviation never exceeded 10% of the mean value.

Figure 3 Light-microscopy photographs of extracellular adenosine production of LLC-MK₂ cells as determined cytochemically

Cells were grown on chamber slides and incubated with 40 nM [α -³²P]ATP (specific radioactivity 800 Ci/mmol) as substrate. CeCl₃ in a final concentration of 0.2 mM was added as a phosphate-capturing agent in the lower panel. Radioactive deposits were visualized using autoradiographic emulsion. Magnification $\times 500$. Bar = 20 μ m.

agent (Figure 3). This method indirectly detects the adenosine produced by complexing the radioactive phosphate released from ATP at the cell surface with Ce³⁺ ions [35].

In an attempt to discriminate between the effect of ATP and the effect of adenosine on cell proliferation, we blocked ATP hydrolysis using inhibitors of the different enzymes involved in extracellular adenosine production. Blocking ecto-ATPase with 1 mM suramin or 1 mM RB-2 caused a significant reduction of the ATP-mediated proliferative effect (Table 1). From these results it can be calculated that the proliferation caused by 100 μ M ATP is inhibited by 71% by suramin and by 84% by RB-2. The inhibition of the ecto-ATPase activity by the same concentrations of suramin and RB-2, as measured biochemically, was 73% and 77% respectively [35]. As determined by Trypan Blue stain cell counting, suramin and RB-2 were shown not to influence the viability of the cells at the concentrations and time periods used in the experiments (viability $105 \pm 21\%$ and $106 \pm 22\%$ respectively, $n = 16$).

In line with these results, a concentration of 100 μ M of non-hydrolysable ATP-analogues, adenosine 5'-[α,β -methylene]-triphosphate and 2-methylthio-ATP, which are in addition P_{2x}- and P_{2y}-purinoceptor agonists, showed no influence on the

Table 1 Influence of enzyme inhibitors on the [*methyl*-³H]thymidine incorporation in LLC-MK₂ cells

Cells were made quiescent by incubation in serum-free medium for 48 h, after which the inhibitors were added at the indicated concentrations. ATP (100 μ M) was used as a stimulating agent and [*methyl*-³H]thymidine as a proliferation marker. After 24 h of incubation, the incorporated radioactivity was counted. The results are expressed as percentage stimulation, compared with the incorporation of radioactivity in the presence of the inhibitor without ATP. Data are mean values ($\pm$ S.D.) of six independent measurements from a typical experiment.

Inhibitor	Stimulation (%)
No inhibitor	154 $\pm$ 16
1 mM Suramin	44 $\pm$ 4
1 mM RB-2	24 $\pm$ 8
100 μ M Adenosine 5'-[α,β -methylene]-diphosphate	45 $\pm$ 2
1 mM Levamisole	102 $\pm$ 15
1 mM Levamisole + 100 μ M adenosine 5'-[α,β -methylene]-diphosphate	11 $\pm$ 1

Table 2 Influence of different nucleosides and nucleoside triphosphates on the [*methyl*-³H]thymidine incorporation in LLC-MK₂ cells

Starvation of the cells was induced by incubating them for 48 h in serum-free medium. Then 100 μ M of the nucleoside triphosphate, or the nucleoside, with or without 100 μ M of adenosine was added, together with [*methyl*-³H]thymidine. After 24 h at 37 °C, [*methyl*-³H]thymidine incorporation into DNA was quantified by liquid scintillation counting. Presented data are mean values ($\pm$ S.D.) for six independent measurements from a typical experiment ($P < 0.001$ for all samples, as compared with control containing no stimulatory agent). In all cases when adenosine was combined with ATP or other nucleosides, no statistically significant differences could be shown when the effect of a combination was compared with the effect of at least one of the agents used in the particular combination.

	Stimulation (%)	
	Without adenosine	With adenosine
ATP	172 $\pm$ 15	204 $\pm$ 4
Adenosine	100 $\pm$ 4	
GTP	55 $\pm$ 3	
Guanosine	66 $\pm$ 3	124 $\pm$ 16
ITP	70 $\pm$ 8	
Inosine	92 $\pm$ 1	94 $\pm$ 2
UTP	-11 $\pm$ 0	
Uridine	-40 $\pm$ 1	-41 $\pm$ 9
CTP	-28 $\pm$ 3	
Cytidine	-51 $\pm$ 6	-49 $\pm$ 11

cellular proliferation (stimulation $8 \pm 1\%$ and $-1 \pm 0\%$ respectively, $n = 6$).

Blocking AMP hydrolysis by either adenosine 5'-[α,β -methylene]-diphosphate (5'-nucleotidase) or levamisole (alkaline phosphatase), caused partial inhibition of the proliferative effect of ATP. When both inhibitors of AMP hydrolysis were applied simultaneously, the stimulatory effect of 100 μ M ATP on cell proliferation was almost completely lost. In contrast, these inhibitors did not influence the proliferative response to adenosine (results not shown).

We also investigated whether the proliferative effect could be achieved by other naturally occurring nucleosides and nucleotides. All purine nucleosides and nucleotides tested, had significant positive effects on cell proliferation, with the strongest response being observed with adenosine and ATP. In contrast to these findings, pyrimidine nucleosides and nucleotides significantly inhibited cell proliferation (Table 2). Inhibition of ecto-ATPase, 5'-nucleotidase and alkaline phosphatase markedly

diminished the effect of the pyrimidine as well as the purine nucleoside triphosphates on cell proliferation (results not shown).

In addition, we studied the effects of the simultaneous presence of different nucleotides and nucleosides. When ATP and adenosine were present together in the cell culture medium, the proliferative effect was not additive (Table 2). Similar results were obtained when adenosine was added together with either guanosine or inosine. However, the simultaneous presence of equimolar concentrations of adenosine and pyrimidine nucleosides (uridine or cytidine) resulted in an inhibitory effect, similar to that observed when only the pyrimidine nucleoside was present.

Since adenosine-containing dinucleotides also exert various biological activities, including cell proliferation [14,15], we examined a possible influence of AP₄A and AP₅A on LLC-MK₂ cells. Both AP₄A and AP₅A showed a stimulatory proliferative effect, similar to that of adenosine, but at much lower concentrations (Table 3). We were unable to show any inorganic phosphate release, as measured spectrophotometrically, when LLC-MK₂ cells were incubated with AP₄A and AP₅A, suggesting that these diadenosine polyphosphates are not hydrolysed. With the spectrophotometric method used, amounts of released phosphate that are 20 times lower than those measured with 1 mM of ATP as substrate, can be detected.

In order to prove that the observed effects on cell proliferation are exclusively due to extracellular nucleosides, we blocked the nucleoside uptake with 10 μ M dipyridamole, a specific nucleoside transporter antagonist. Dipyridamole also inhibits the uptake of [*methyl*-³H]thymidine ($92 \pm 7\%$ inhibition, $n = 5$). Therefore the relative change in cell number was determined with a method relying on the use of Alamar Blue as a redox indicator. We found that dipyridamole did not reduce the proliferation caused by either 100 μ M adenosine or ATP. Also the inhibitory effect of CTP was not reduced by dipyridamole (Table 4). These results suggest that the extracellular presence of the nucleosides tested is responsible for the observed effects on proliferation.

In an attempt to determine which type of purinoceptor is involved in the proliferation of LLC-MK₂ cells, we tested *N*⁶-cyclopentyladenosine and *N*⁶-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine respectively as specific A₁- and A₂-receptor agonists. No stimulating effect was observed when these substances were tested at concentrations ranging from 1 nM to 10 μ M. Addition of 8-(*p*-sulphophenyl)theophylline, an antagonist of adenosine receptors, at concentrations up to 100 μ M, had no influence on the proliferative effect of either 100 μ M ATP or adenosine (results not shown).

In most cases, adenosine receptors mediate their effect on cell proliferation through a mechanism which involves cyclic AMP as a second messenger. Therefore, we determined intracellular cyclic AMP levels after stimulation with 100 μ M adenosine or cytidine. With 100 μ M adenosine, no increase of the intracellular cyclic AMP concentration could be found. Also, 100 μ M cytidine did not increase the cyclic AMP content of the cells. Inhibition of cyclic AMP breakdown, by blocking phosphodiesterase with 20 μ M RO 20-1724, did not alter the effect of adenosine or cytidine. Furthermore the increase in cyclic AMP levels due to 10 μ M forskolin, a stimulator of adenylate cyclase, was not influenced by the additional presence of either adenosine or cytidine. Cells were also incubated with 8-bromo-cyclic AMP, which is taken up by the cells and transformed into cyclic AMP, and therefore could potentially stimulate proliferation. 8-Bromo-cyclic AMP (100 μ M) did not cause any effect on the proliferation of the cells (stimulation $14 \pm 0.3\%$, $n = 7$). Similarly, 8-bromo-cyclic GMP (100 μ M) was found to be inactive in stimulating the proliferation (stimulation $10 \pm 1\%$,

Table 3 Effect of AP₄A and AP₅A on the incorporation of [*methyl*-³H]thymidine in LLC-MK₂ cells

Starvation of the cells was induced by growing them for 2 days in serum-free medium. AP₄A and AP₅A were added (10 μ M, 1 μ M, 100 nM) together with [*methyl*-³H]thymidine and the cells were incubated for one more day. The amount of [*methyl*-³H]thymidine incorporated in the cellular DNA was quantified. Results are from one representative experiment in quadruplicate (mean values $\pm$ S.D.; **P* < 0.05)

	Stimulation (%)		
	10 μ M	1 μ M	0.1 μ M
AP ₄ A	104 $\pm$ 15*	73 $\pm$ 6*	32 $\pm$ 7
AP ₅ A	98 $\pm$ 37*	48 $\pm$ 3*	21 $\pm$ 4

Table 4 Effect of 10 μ M dipyridamole on the number of LLC-MK₂ cells

Cells were grown in NBSC-free DMEM for 48 h. ATP (100 μ M), CTP (100 μ M), adenosine (100 μ M) and NBSC (10%) were added, without or with 10 μ M dipyridamole. After 72 h, the relative cell number was determined by the use of Alamar Blue. Results from a typical experiment are given in percentage stimulation, compared with a blank containing no stimulating agents.

	Stimulation (%)	
	Without dipyridamole	With dipyridamole
Adenosine	48 $\pm$ 13	66 $\pm$ 8
ATP	72 $\pm$ 6	77 $\pm$ 13
CTP	-14 $\pm$ 6	-5 $\pm$ 2

n = 5). The possibility that IP₃ acts as a second messenger of the observed proliferative effect was also checked. We found that the IP₃ concentration was not influenced either by proliferation stimulatory agents like 100 μ M ATP or adenosine or by proliferation inhibitory agents like 100 μ M CTP or cytidine (concentrations of IP₃ were 1–5 % lower than control). These findings are strengthened by the fact that staurosporine, a protein kinase C inhibitor, did not influence the proliferative effects of any of the nucleosides (results not shown).

In order to find out whether influx of extracellular Ca²⁺ ions could be responsible for the proliferating stimulus in LLC-MK₂ cells, we added EGTA to the medium. We found that EGTA, up to 1 mM, was not capable of blocking any positive or negative proliferative effect.

In an attempt to check for a possible participation of G-proteins in a mechanism by which the nucleosides mediate cell proliferation, we studied the effect of pertussis and cholera toxins. Pre-incubation of the cells for 4 to 6 h with the toxins, did not influence the proliferative effect of the nucleosides.

Finally we examined a possible involvement of the Na⁺/H⁺-exchanger in the process of cell proliferation by extracellular nucleosides. We compared the effect of various nucleosides in the presence of 50 μ M 5-(*N,N*-dimethyl)amiloride, an inhibitor of the Na⁺/H⁺-exchanger. Blocking the exchanger significantly decreased the proliferative effect of purine nucleosides (adenosine, -28 $\pm$ 2 %; guanosine, -38 $\pm$ 1 %; and inosine, -35 $\pm$ 2 %, *n* = 6; *P* < 0.01). In the case of uridine and cytidine, 5-(*N,N*-dimethyl)amiloride significantly increased the inhibiting effect of the nucleosides (18 $\pm$ 1 % and 53 $\pm$ 1 % respectively, *n* = 6; *P* < 0.01).

DISCUSSION

In this study, we investigated the effect of extracellular nucleosides and nucleotides on five randomly chosen cell lines. Adenosine and ATP were shown to positively affect proliferation of three cell lines: LLC-MK₂, Cos-7 and GM00637F. We focused our attention on LLC-MK₂ cells for two reasons. First, these cells showed a high proliferative response to externally added ATP and adenosine, and secondly, they express a high activity of ecto-enzymes with nucleotide-dephosphorylating capacities [35]. When adenosine, AMP, ADP and ATP were tested for proliferation effects, ATP was shown to influence the cellular proliferation to the highest extent, followed by ADP, AMP and adenosine.

The proliferative effect we observed is caused by extracellular adenine nucleotides or adenosine, since blocking the nucleoside transporter had no effect on the stimulation caused by the nucleosides or nucleotides. This conclusion is further supported by the fact that we observed a fully developed proliferative effect after exposure of the cells to adenosine or ATP for only 10 min (results not shown).

Our results do not correspond with the known pharmacological properties of P₂-purinoceptors [1,3,36]. These receptors bind ATP and ADP as the active ligands, whereas AMP and adenosine are pharmacologically inactive. Evidence against the involvement of P₂-purinoceptors in the observed proliferative effect comes from the experiment in which different steps of the extracellular ATP dephosphorylation were blocked. Together with the ineffectiveness of adenosine 5'-[α,β -methylene]-triphosphate and 2-methylthio-ATP in influencing the proliferation of LLC-MK₂ cells, these results rule out the possibility of a major involvement of P₂-purinoceptors and clearly show that adenosine is causing the proliferation of LLC-MK₂ cells. Our results are partially in disagreement with the study of Van Daele et al. [7], who found evidence for the presence of a P₂-purinoceptor, as well as for an unusual class of adenosine receptors being involved in the proliferation of aortic endothelial cells. Our present results point out the possible importance of ecto-nucleotidase enzymes in the control of cell proliferation mediated by extracellular nucleotides, as also suggested by Slivinskii et al. [37]. These authors show that alkaline phosphatase, in addition to 5'-nucleotidase, can be an important pathway for the extracellular production of adenosine. Also, it was shown that the presence of ecto-ATPase activity has a marked effect on the apparent potency of ATP on P₂-purinoceptors, indicating that ecto-ATPase could be a key element in regulation (termination) of the cellular response to ATP [38].

It is interesting to note that adenosine itself stimulates 5'-nucleotidase in rat mesangial cells [39]. In different cells its activity increases during the exponential growth phase of the cells in culture and is significantly down-regulated once the cells reach confluency [40].

We found that all observed effects were due to the nucleosides; while the purine nucleosides stimulated, the pyrimidine nucleosides inhibited cell proliferation. Similar to our results, Kartha and Toback [41] showed that purine nucleotides, but not pyrimidine nucleotides, stimulate cell proliferation of BSC-1 cells. Furthermore GTP and guanosine have been reported to cause proliferation of different cell types [42]. In contrast to these findings, guanosine induces necrosis of cultured aortic endothelial cells [43]. UTP and uridine inhibit the proliferation of LLC-MK₂ cells and of endothelial cells [7]. Uridine also inhibits the growth of neuroblastoma cells [19]. Furthermore, purine nucleosides and nucleotides stimulate chick astrocyte proliferation *in vitro*, while pyrimidine nucleosides and nucleotides are inactive [44].

The lack of a fully additive effect for adenosine and ATP argues against two separate modes of action for ATP and adenosine. This is in agreement with the experiments where inhibition of ATP hydrolysis blocked the proliferative effect of ATP. The greater response to ATP can be explained by a stimulatory effect of ATP on the proliferation due to adenosine. Also, no additive or synergistic effect could be seen with combinations of two purine nucleosides, suggesting that all purine nucleosides may act on a common putative purinoceptor. When purine nucleosides were combined with pyrimidine nucleosides, the inhibitory effect of the pyrimidine nucleosides completely overruled the stimulating effect of the purine nucleosides.

Diadenosine polyphosphates are involved in the physiological control of blood pressure [45] and regulation of neutrophil function [46]. We found that AP_4A and AP_5A were more potent than adenosine or ATP in stimulating cell proliferation. AP_4A and AP_5A presumably do not penetrate normal, intact cell membranes, suggesting that the proliferative effect we observed could be due to direct binding of these compounds to receptors of the P_{2d} type [3]. It is also possible that in some other cell types AP_4A and AP_5A influence cell proliferation through a dual effect, binding to P_{2d} -purinoceptors and binding of ATP, ADP, AMP or adenosine, produced as degradative products of diadenosine polyphosphates, to other classes of purinoceptors [10,47,48]. With the analytical method used, we obtained no evidence that LLC-MK₂ cells can hydrolyse these diadenosine phosphates. Therefore they cannot act as precursors for adenosine. Due to the structural differences between the diadenosine polyphosphates and adenosine, it seems highly unlikely that they exert their proliferative effect through binding to a common receptor. A receptor for diadenosine phosphates has been identified and biochemically characterized in different cell types [49,50]. Our results suggest the presence of such a receptor on LLC-MK₂ cells, making it likely for AP_4A and AP_5A to act as regulators of cell proliferation.

The question remains as to what kind of receptors mediate the proliferative effect of nucleosides. We found no evidence that the effect of adenosine is mediated by either A_1 - or A_2 -receptors, as was described previously by Kartha and Toback [41] for BSC-1 cells. According to our results, it seems unlikely that cyclic AMP, cyclic GMP, IP_3 , protein kinase C or Ca^{2+} mediates the proliferation triggered by extracellular adenosine. It is also interesting to note that, in monkey kidney epithelial cells, cyclic AMP does not stimulate proliferation [41]. The conclusion that the effect of adenosine may be mediated by an unusual class of low-affinity nucleoside receptor is in good agreement with the study of Van Daele et al. [7] with endothelial cells. Our observation that both pertussis and cholera toxins did not alter the proliferative effects of the various nucleosides or nucleotides, supports this conclusion and suggests that the signal transduction is probably not linked to pertussis or cholera toxin-sensitive G-proteins.

According to our results, it is possible to propose that all purine nucleosides act on one class of putative receptors. Characterization of the inhibiting proliferative effects of the pyrimidine nucleosides suggests that they also might regulate this function upon binding to a new class of putative pyrimidinergic receptors. Currently it has been accepted that nucleosides other than adenosine are ineffective on P_1 -purinoceptors, while nucleoside triphosphates other than ATP, UTP [3] and exceptionally GTP [51] are inactive on P_2 -purinoceptors.

Activation of the Na^+/H^+ -exchanger is an early event generally considered to be necessary for stimulation of protein synthesis and cell-cycle progression. Our results show that in LLC-MK₂

cells the Na^+/H^+ -exchanger might indeed be involved in the regulation of cell proliferation by nucleosides. Since the involvement of cyclic AMP, cyclic GMP, IP_3 , protein kinase C or Ca^{2+} is excluded, the dependence of the effects on a functional Na^+/H^+ -exchanger supports the possibility that the putative receptors may belong to the protein tyrosine kinase or a related receptor group [52]. It was shown that endothelial cells stimulated by extracellular ATP undergo intracellular alkalinization as a result of subsequent activation of the Na^+/H^+ -exchanger [53]. Recently it was shown that ATP and UTP trigger the activation of mitogen-activated protein kinase cascade and that this may be responsible for the potent mitogenic activity of both nucleotides [6].

In summary, we have presented evidence that the proliferation of LLC-MK₂ cells is influenced by nucleosides formed through dephosphorylation of extracellular nucleotides by ecto-enzymes, which can therefore act as regulators of cellular proliferation. The proliferative effect of nucleosides is of extracellular origin with purine nucleosides causing stimulation of cell proliferation and the pyrimidine nucleosides causing inhibition of cell proliferation. The Na^+/H^+ -exchanger plays a role in both pathways for regulating cell proliferation. These pathways are not completely independent of each other, since the effect of pyrimidine nucleosides overrules the effect of purine nucleosides. Furthermore, this control seems not to be mediated by any of the known P_1 - or P_2 -purinoceptors.

We wish to thank Dr. L. Kupers and Dr. P. Stinissen for the use of laboratory facilities and the staff of the Dr. Willems Institute for their co-operation. We also thank Dr. P. Janssen for his valuable advice with the statistical calculations.

REFERENCES

- Dubyak, G. R. and El-Moatassim, C. (1993) *Am. J. Physiol.* **265**, C577–C606
- Jacobson, K. A., van Galen, P. J. M. and Williams, M. (1992) *J. Med. Chem.* **35**, 407–422
- Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P. and Williams, M. (1994) *Pharmacol. Rev.* **46**, 143–156
- Huang, N.-N., Wang, D.-J., Gonzalez, F. A. and Heppel, L. A. (1991) *J. Cell. Physiol.* **146**, 483–494
- Wang, D.-J., Huang N.-N., Gonzalez, F. A. and Heppel, L. A. (1991) *J. Cell. Physiol.* **146**, 473–482
- Huwiler, A. and Pfeilschifter, J. (1994) *Br. J. Pharmacol.* **113**, 1455–1463
- Van Daele, P., Van Coevorden, A., Roger, P. P. and Boeynaems, J.-M. (1992) *Circ. Res.* **70**, 82–90
- Malam-Souley, R., Campan, M., Gadeau, A.-P. and Desgranges, C. (1993) *Am. J. Physiol.* **264**, C783–C788
- Erlinge, D., Yoo, H., Edvinsson, L., Reis, D. J. and Wahlestedt, C. (1993) *Am. J. Physiol.* **265**, H1089–H1097
- Erlinge, D., You, J., Wahlestedt, C. and Edvinsson, L. (1995) *Eur. J. Pharmacol.* **289**, 135–149
- Huang, N.-N., Wang, D. and Heppel, L. A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7904–7908
- Ishikawa, S.-E., Kawasumi, M., Kusaka, I., Komatsu, N., Iwao, N. and Saito, T. (1994) *Biochem. Biophys. Res. Commun.* **202**, 234–240
- Greenberg, S., Di Virgilio, F., Steinberg, T. H. and Silverstein, S. C. (1988) *J. Biol. Chem.* **263**, 10337–10343
- Hotta, T., Asai, K., Takeda, N., Yoshizumi, H., Tatematsu, A., Nakanishi, K., Eksiglu, Y. Z., Isobe, I. and Kato, T. (1993) *J. Neurochem.* **61**, 1430–1437
- Andersson, M. (1989) *Int. J. Biochem.* **21**, 707–714
- Meininger, C. J., Schelling, M. E. and Granger, H. J. (1988) *Am. J. Physiol.* **255**, H554–H562
- Meininger, C. J. and Granger, H. J. (1990) *Am. J. Physiol.* **258**, H198–H206
- Ethier, M. F., Chander, V. and Dobson, J. G., Jr. (1993) *Am. J. Physiol.* **265**, H131–H138
- Hotta, T., Asai, K., Takeda, N., Tatematsu, A., Nakanishi, K., Eksiglu, Y. Z., Isobe, I. and Kato, T. (1993) *J. Neurochem.* **60**, 312–319
- Rozengurt, E. (1982) *Exp. Cell. Res.* **139**, 71–78

- 21 Weisman, G. A., Lustig, K. D., Lane, E., Huang, N.-N., Belzer, I. and Friedberg, I. (1988) *J. Biol. Chem.* **263**, 12367–12372
- 22 Jackson, J. A. and Carlson, E. C. (1992) *Am. J. Physiol.* **263**, H634–H640
- 23 Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen, J.-J., Dumont, J. E., Vassart, G. and Schiffmann, S. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1169–1178
- 24 Yang, S., Cheek, D. J., Westfall, D. P. and Buxton, I. L. O. (1994) *Circ. Res.* **74**, 401–407
- 25 Gordon, J. L. (1986) *Biochem. J.* **233**, 309–319
- 26 Wardell, C. F. and Cunnane, T. C. (1994) *Br. J. Pharmacol.* **111**, 975–977
- 27 Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., Guidotti, G. and Cantiello, H. F. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 312–316
- 28 Cantiello, H. F., Prat, A. G., Reisin, I. L., Ercole, L. B., Abraham, E. H., Amara, J. F., Gregory, R. J. and Ausiello, D. A. (1994) *J. Biol. Chem.* **269**, 11224–11232
- 29 Reisin, I. L., Prat, A. G., Abraham, E. H., Amara, J. F., Gregory, R. J., Ausiello, D. A. and Cantiello, H. F. (1994) *J. Biol. Chem.* **269**, 20584–20591
- 30 Grierson, J. P. and Meldolesi, J. (1995) *J. Biol. Chem.* **270**, 4451–4456
- 31 Gordon, E. L., Pearson, J. D. and Slakey, L. L. (1986) *J. Biol. Chem.* **261**, 15496–15504
- 32 Gordon, E. L., Pearson, J. D., Dickinson, E. S., Moreau, D. and Slakey, L. L. (1989) *J. Biol. Chem.* **264**, 18986–18992
- 33 Culic, O., Sabolic, I. and Zanich-Grubisic, T. (1990) *Biochim. Biophys. Acta* **1030**, 143–151
- 34 Lebel, D., Poirier, G. G. and Beaudoin, A. R. (1978) *Anal. Biochem.* **85**, 86–89
- 35 Culic, O., Lemmens, R., Teuchy, H. and Vanduffel, L. (1995) *Histochem. J.* **27**, 555–564
- 36 O'Connor, S. E., Dainty, I. A. and Leff, P. (1991) *Trends Pharmacol. Sci.* **12**, 137–141
- 37 Slivinskii, G. G., Bushneva, I. A. and Magda, I. N. (1993) *Tsitologiya* **35**, 94–100
- 38 Crack, B. E., Beukers, M. W., McKechnie, K. C. W., Ijzerman, A. P. and Leff, P. (1994) *Br. J. Pharmacol.* **113**, 1432–1438
- 39 Stefanovic, V., Vlahovic, P., Savic, V., Ardaillou, N. and Ardaillou, R. (1993) *FEBS Lett.* **331**, 96–100
- 40 Turnay, J., Olmo, N., Risse, G., von der Mark, K. and Lizarbe, M. A. (1989) *In Vitro Cell. Dev. Biol.* **25**, 1055–1061
- 41 Kartha, S. and Toback, F. G. (1985) *Am. J. Physiol.* **249**, F967–F972
- 42 Rathbone, M. P., Middlemiss, P. J., Gysbers, J. W., DeForge, S., Costello, P. and Del Maestro, R. F. (1992) *In Vitro Cell. Dev. Biol.* **28**, 529–536
- 43 Han, Z. and Wyche, J. H. (1994) *Am. J. Pathol.* **145**, 423–427
- 44 Christjanson, L. J., Middlemiss, P. J. and Rathbone, M. P. (1993) *Glia* **7**, 176–182
- 45 Schlüter, H., Offers, E., Brüggemann, G., Van Der Giet, M., Tepel, M., Nordhoff, E., Karas, M., Spieker, C., Witzel, H. and Zidek, W. (1994) *Nature (London)* **367**, 186–188
- 46 Gasmil, L., McLennan, A. G. and Edwards, S. W. (1994) *Biochem. Biophys. Res. Commun.* **202**, 218–224
- 47 Luthje, J. and Oglivie, A. (1985) *Eur. J. Biochem.* **149**, 119–127
- 48 Goldman, S. J., Gordon, E. L. and Slakey, L. L. (1986) *Circ. Res.* **59**, 362–366
- 49 Hilderman, R. H., Martin, M., Zimmerman, J. K. and Pivorun, E. B. (1991) *J. Biol. Chem.* **266**, 6915–6918
- 50 Walker, J., Bossman, P., Lackey, B. R., Zimmerman, J. K., Dimmick, M. A. and Hilderman, R. H. (1993) *Biochemistry* **32**, 14009–14014
- 51 Vuorinen, P., Wu, X., Arvola, P., Vapaatalo, H. and Pörsti, I. (1994) *Br. J. Pharmacol.* **112**, 71–74
- 52 Dumont, J. E., Jauniaux, J.-C. and Roger, P. P. (1989) *Trends Biochem. Sci.* **14**, 67–71
- 53 Kitazono, T., Takeshige, K., Cragoe, E. J., Jr. and Minekami, S. (1989) *Biochim. Biophys. Acta* **1013**, 152–158