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Interleukin-6 Enhances Expression of Adenosine A_1 Receptor mRNA and Signaling in Cultured Rat Cortical Astrocytes and Brain Slices

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The inhibitory neuromodulator adenosine is released in the brain in high concentrations under conditions of exaggerated neuronal activity such as ischemia and seizures, or electroconvulsive treatment. By inhibiting neural overactivity, adenosine counteracts seizure activity and promotes neuronal survival. Since stimulation of adenosine A_{2b} receptors on astrocytes induces increased synthesis and release of interleukin-6, which also exerts neuroprotective effects, we hypothesized that the effects of interleukin-6 and of adenosine might be related. We report here that stimulation with interleukin-6 of cultured astrocytes, of cultured organotypic brain slices from newborn rat cortex, and of freshly prepared brain slices from rat cortex induces a concentration- and time-dependent upregulation of adenosine A_1 receptor mRNA. This increased adenosine A_1 receptor mRNA expression is accompanied in astrocytes by an increase in adenosine A_1 receptor-mediated signaling via the phosphoinositide-dependent pathway. Since upregulation of adenosine A_1 receptors leads to increased neuroprotective effects of adenosine, we suggest that the neuroprotective actions of interleukin-6 and adenosine are related and might be mediated at least in part through upregulation of adenosine A_1 receptors. These results may be of relevance for a better understanding of neuroprotection in brain damage but also point to a potential impact of neuroprotection in the mechanisms of the antidepressive effects of chronic carbamazepine, electroconvulsive therapy, and sleep deprivation, which are all accompanied by adenosine A_1 receptor upregulation. ***[Neuropsychopharmacology 24:86–96, 2001]***

KEY WORDS: *Adenosine A_1-receptor; Interleukin-6; Neuroprotection; Astrocytes; Organotypic brain slices*

Adenosine, as a metabolite of ATP, nature's general energy source, has acquired early in evolution the general function of signaling and counteracting a dysbalance of energy supply and demand (Newby 1984). In the brain, adenosine acts as an inhibitory neuromodulator, which is released under conditions of neuronal activity and reduces this activity by inhibition of transmitter release and postsynaptic hyperpolarisation. Brain damage, e.g., by stroke, ischemia, and seizures leads to a pronounced increase in extracellular adenosine (for review see Rudolphi and Schubert 1996) which counteracts seizure activity (Dragunow 1988) and promotes neuronal survival (Deckert and Gleiter 1994; Picano and Abbracchio 1998; Dux et al. 1990).

In addition to its role as an endogenous anticonvulsant and neuroprotective agent, adenosine is now recognized as an important regulator of sleep and wakefulness (for review see Porkka-Heiskanen 1999).

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Adenosine's actions in the brain and elsewhere in the body are mediated by at least four subtypes of extracellular receptors, A_1, A_{2a}, A_{2b}, and A_3, which have been cloned, are all coupled to guanine nucleotide binding proteins (G proteins), and have different patterns of tissue expression (for review see Olah and Stiles 1995). In the brain, A_1 receptors are widely distributed and are particularly prominent in the hippocampus, striatum, and neocortex. A_{2a} receptors are largely restricted to dopamine-rich areas such as the caudate, putamen, nucleus accumbens, and olfactory tubercle and are likely involved in the regulation of dopamine signaling, whereas A_{2b} receptors are widely distributed in the brain and may be predominantly localized on astrocytes (Williams 1995). Adenosine A_1- and A_2-receptor subtypes were originally distinguished by their differential effects on adenylyl cyclase (van Calker et al. 1978, 1979), but more recent findings have revealed coupling of adenosine receptors to other signaling systems including the phosphoinositol system, potassium, and calcium channels (for review see Fredholm et al. 1994; Ralevic and Burnstock 1998).

Adenosine mediates neuroprotection by inhibiting presynaptically the release of several neurotransmitters including the excitatory neurotransmitter glutamate (Ribeiro 1995). Postsynaptically, adenosine increases conductances of various K^+- and Cl^- -channels, which hyperpolarize the membrane potential and counteract a transmitter induced depolarisation with a subsequent reduction of Ca^{2+} -influx and stabilization of the Mg^{2+} blockage of NMDA receptors (Rudolphi et al. 1992a,b; Rudolphi and Schubert 1996). Most, if not all, of these actions of adenosine are mediated via the adenosine A_1 receptor, whereas the role of the other adenosine receptor subtype in neuroprotection is less clear (Schubert et al. 1997; Abbracchio and Cattabeni 1999; Heurteaux et al. 1995). Accordingly, upregulation of adenosine A_1 receptors by chronic treatment with A_1-antagonists increases the neuroprotective effect of adenosine (Sutherland et al. 1991; Rudolphi et al. 1992a,b).

Upregulation of adenosine A1-receptors is also observed after treatments that exert antidepressive effects in humans such as seizures and electroconvulsive treatment (ECT) (Newman et al. 1984; Gleiter et al. 1989; Angelatou et al..1993; Pagonopoulou et al. 1993), REM sleep deprivation (Yanik and Radulovacki 1987), and chronic treatment with carbamazepine (Daval et al. 1989; Biber et al. 1999; van Calker et al. 2000). While the upregulation by carbamazepine of adenosine A_1 receptors is readily comprehensible from carbamazepine's selective antagonistic effects on A_1 receptors (Clark and Post 1989; van Calker et al. 1991; Biber et al. 1996), the upregulation of A_1 receptors after ECT or sleep deprivation presents a paradox, since the increase in extracellular adenosine concentrations under these conditions (Huston et al. 1996; Porkka-Heiskanen et al. 1997; Dragunow 1988) should rather downregulate adenosine A_1 receptors (Hettinger et al. 1998; Ruiz et al. 1996).

We have, therefore, hypothesized that adenosine might induce the synthesis and release of a factor that promotes the upregulation of A_1 receptors and, thus, counteracts their downregulation by adenosine. As one potential candidate for such a factor, we considered the cytokine interleukin-6 (Il-6) since we and others have recently shown that the synthesis of Il-6 in astrocyte-like cells is stimulated by adenosine via A_{2b}-receptors (Fiebich et al. 1996; Schwaninger et al. 1997). Furthermore, Il-6 appeared a likely candidate for such a function due to several unique properties: it is, like adenosine, synthesized and released in brain under pathological conditions such as ischemia (Loddick et al. 1998) and excitatory overstimulation (Schiefer et al. 1998) and in brain cell cultures after hypoxia (Maeda et al. 1994). IL-6 can be produced *in vivo* and *in vitro* by different brain cell types including microglia (Murphy et al. 1998), astrocytes (Fiebich et al. 1996; Schwaninger et al. 1997), and neurons (Schobitz et al. 1993; Gadient and Otten 1996; Maerz et al. 1998). Furthermore, similar to adenosine, IL-6 appears to have also neuroprotective properties, the mechanisms of which have remained elusive (for review see Groul and Nelson 1997; Gadient and Otten 1997).

IL-6 promotes the survival of neurons both under basal culture conditions (Kushima et al. 1992a; Kushima and Hatanaka 1992b) and under conditions of ischemia (Matsuda et al. 1996; Loddick et al. 1998) or induced exitotoxicity (Yamada and Hatanaka 1994; Toulmond et al. 1992). Moreover, IL-6 was also found to be neuroprotective *in vivo* upon cryolesion induced brain damage, as shown recently in IL-6 deficient mice (Penkowa et al. 1999). However, similarly to other cytokines (Munoz-Fernandez and Fresno 1998), Il-6 may exert both beneficial and detrimental activities in neuronal tissue depending on yet undefined factors (Campbell 1998; Gadient and Otten 1997; Merrill and Jonakait 1995).

We report here that stimulation of astrocytes and brain slices with IL-6 induces a pronounced increase of adenosine A_1 receptor mRNA expression and that in astrocytes this increase is associated with an upregulation of adenosine A_1 receptor mediated phosphoinositol signaling.

METHODS

Reagents were purchased from the following sources: Cyclopentyladenosine (CPA) and phenylephrine from Research Biochemical, Inc. (RBI distributed by Sigma, Deisenhofen, Germany); Endotoxin free rat interleukin-6 (IL-6) from PeproTech (London, UK); Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution, and horse serum from Sigma (Deisenhofen, Germany); Fetal calf serum from Boehringer (Mannheim, Germany); Scintillation fluid (rotiszint ecoplus) from

Roth (Karlsruhe, Germany); Dowex anion exchanger (formiate form AG 1 × 8) from BioRad (Munich, Germany); [^{3}H]-*myo*-inositol and [^{32}P]dCTP from Amersham and Buchler (Braunschweig, Germany); Bst E II from Pharmacia (Freiburg, Germany); Dynabeads from Dynal AG (Hamburg, Germany); Murine Moleney Leukemia Virus Reverse Transcriptase (M-MLV RT) and T4 ligase from Gibco BRL Life Technologies (Eggenstein, Germany); Taq Polymerase from InViTek (Berlin, Germany); TA cloning kit from Invitrogen (Leek, The Netherlands); Random priming kit Prime IT II from Stratagene (Heidelberg, Germany). The cDNA probe encoding β-actin was a generous gift from Dr. G. Finkenzeller (Institute for Tumor Biology, Freiburg, Germany)

Cell Cultures

Astrocyte cultures were established as described previously (Biber et al. 1997). In brief, rat cortex was dissected under sterile conditions from newborn Wistar rats (< 1d). The brain tissue was gently dissociated by trituration in Dulbecco's phosphate buffered saline and filtered through a cell strainer (70 μm Ø, Falcon) into Dulbecco's modified Eagle's medium (DMEM). After two washing steps (200g for 10 min), cells were seeded into 24-well-dishes (Falcon) (5×10^5 cells/well). For total RNA and mRNA preparation, cells were grown in 6-well dishes (Falcon) (2×10^6 cells/well) and standard dishes (Falcon, 10 cm Ø) (8×10^6 cells/dish), respectively.

Cultures were maintained for four weeks in DMEM containing 10% foetal calf serum with 0.01% penicillin and 0.01% streptomycin in a humidified atmosphere (5% CO_2) at 37°C. Culture medium was changed on the second day after preparation and every six days thereafter.

Organotypic Slice Cultures

Cortex was dissected from neonatal rats and slices (350 μm) were cut under aseptic conditions using a McIlwain tissue chopper. The slices were cultured as described by Frotscher and Heimrich (1993). Slices were placed on Millipore membranes (Millicell-CM) that were transferred into 6-well dishes containing 1ml medium of the following composition [50% (vol/vol) minimal essential medium; 25% (vol/vol) Hank's balanced salt solution; and 25% (vol/vol) inactivated horse serum] containing 2mM glutamine and 0.044% NaHCO3 (final concentration) adjusted to pH 7.3. The slices (three per membrane) were cultured for four days in a humidified atmosphere (5% CO_2) at 37°C before the experiments. The medium was changed on the second day and 1 hour before stimulation.

Brain Slices

Young Wistar rats (13–17-d-old) were killed by decapitation in accordance with institutional guidelines and the cerebral cortices prepared. Slices of the cerebral cortex (300 μm) were cut with a vibratom (DTK 1000; Dosaka (EM Co Ltd, Kyoto, Japan)) and incubated for three hours at 37°C in ringer solution containing the various additions (IL-6 or vehicle) oxygenated with a constant flow of carbogene (95% O_2, 5% CO_2).

Inositolphosphate Determination

Four week-old astrocyte cultures were labelled for 24 h with 1 μCi [^{3}H]-*myo*-inositol in 250μl of culture medium. After three washings with 500 μl incubation buffer (118 mM NaCl, 4.7 mM KCl, 3 mM CaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM Glucose, and 20 mM HEPES; pH 7.4), cells were incubated for 15 min at 37°C in the same buffer supplemented with 10 mM LiCl and stimulated for 20 min with the α_1-adrenergic receptor agonist phenylephrine (100 μM) in the presence or absence of CPA. The reaction was stopped by the addition of ice-cold trichloroacetic acid (TCA; 100% w/v) up to a final concentration of 10% TCA. Dishes were incubated on ice for 30 min.

Separation of inositolphosphates was performed on Dowex anion exchange columns (Formiate Form AG 1 × 8) as described previously (Biber et al. 1997). In brief, TCA was extracted with diethylether (three times) and samples were neutralized to pH 7 with 5 mM disodium tetraborate. Samples were loaded on Dowex columns and after two washing steps (10 ml water; 10 ml 50 mM disodium tetraborate/60 mM ammonium formate) inositolphosphates were eluted with 2 ml of 1 M ammonium formate/0.1 M formic acid. Samples were mixed with 6ml of liquid scintillation fluid and counted. Determinations were performed in triplicate. Data are given as means ± SD and statistical analysis was performed by Students t-test.

Total RNA Extraction

Cells, brain slices and slice cultures were lysed in guanidinium isothiocyanate/mercaptoethanol (GTC) solution and total RNA was extracted according to Chomczynski and Sacchi (1987).

mRNA Extraction and Northern Analysis

Cells were lysed in 500 μl lysis/binding buffer (Dynal, Hamburg, Germany) and mRNA was extracted with 150 μl Dynabeads according to the manufacturers protocol. mRNA (2–3 μg) was eluted in 15 μl water, separated by agarose-formaldehyde gel electrophoresis, blotted onto positively charged Nylon membranes (Pharmacia, Freiburg, Germany), and cross-linked by exposure to 120°C for 30 min. As a marker, 15 μg of total RNA was run on the same gel and stained with ethidium bromide to determine the bands for 18s and

28s ribosomal RNA. The filters were prehybridized in 50% formamide, 0.25 M Na-phosphate buffer, pH 7.2, 0.25 M NaCl, 10 mM EDTA, 200 μg/ml salmon sperm DNA, and 7% SDS at 43°C for 2 h. cDNA probes (as used in Biber et al. (1999)) were labelled with 50 μCi [^{32}P]dCTP (Amersham and Buchler, Braunschweig, Germany) using a random priming kit from Stratagene (Heidelberg, Germany). Unincorporated nucleotides were removed using a nucleotide removal kit from Qiagen.

Overnight hybridization was performed at 43°C, adding the radiolabeled probe to the prehybridization buffer. Membranes were washed in 2 × SSC/0.1% SDS (3 × 20 min) at 60°C and exposed to Kodak XAR film at −80°C for adequate periods of time. For rehybridization, probes were removed by boiling the filter at 95°C in distilled water. Experiments were carried out in triplicate. Band intensities were determined with a gel imaging program (One-DeScan; MWG Biotech, Germany).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse Transcription. In case of cultured astrocytes, 1 μg of total RNA was transcribed into cDNA in a final volume of 25 μl containing 200 U M-MLV RT (Gibco), 40 U RNAse inhibitor (Pharmacia, Freiburg, Germany), 1 μl random hexamers (2.5 nM), 9 μl H_2O, 5 μl 5 × buffer (Gibco), 4 μl DTT (0.1 M), and 5 μl dNTPs (2.5 mM). After 10 min of incubation at 30°C and 60 min at 42°C, the reaction was stopped by heating at 95°C for 5 min. Since the RNA amounts obtained from cultured brain slices were very limited (approximately 0.2 to 1 μg), all RNA was used in the RT. The RT protocol was the same as for RNA from cultured astrocytes.

Potential contamination by genomic DNA were checked for by running the reactions without reverse transcriptase and using S12 primers in subsequent PCR amplifications. Only RNA samples which showed no bands after that procedure were used for further investigation.

Polymerase Chain Reaction. For PCR amplification, the following reagents were added to 2 μl of the RT-reaction: 4 μl MgCl 25 mM, 5 μl 10 × PCR Buffer (InViTek, Berlin, Germany), 4 μl dNTPs 10 mM, 35 μl H_2O, 0.5 μl of each primer, and 0.5 U Taq Polymerase (InViTek). PCR conditions were as follows: 1 min denaturation at 94°C, 1 min primer annealing, and 1.5 min amplification at 72°C. PCR was terminated by an incubation at 72°C for 7 min.

Sequences of oligonucleotide primer pairs and PCR conditions: Adenosine A_1 receptor, (Mahan et al. 1991) No. 55 5′-ATTGCCTTGGTCTCTGTGC and No. 690 5′-CAGCTCCTTCCCGTAGTAC, annealing temperature 59°C, 35 cycles. S12 ribosomal protein (Ayane et al. 1989) No. 49 5′-ACGTCAACACTGCTCTACA and No. 360 5′-CTTTGCCATAGTCCTTAAC, annealing temperature 56°C, 28 cycles. The plateau phase of the PCR-reaction was not reached under these PCR-conditions.

Quantification of PCR-Products. PCR products were quantified as described previously (Biber et al. 1997). In brief; amplified cDNAs were separated on ethidium bromide stained agarose gels and analyzed by use of a gel imaging system (One-DeScan; MWG Biotech, Germany). Arbitrary units of adenosine A_1 receptor mRNA were correlated to arbitrary units of S12 mRNA (= 100%) of the same sample. Data are given as means ± SEM and statistical analysis were performed by Student's t-test.

Competitive RT-PCR

Competitive RT-PCR was used to determine the increase of adenosine A_1 receptor mRNA expression. A deletion product was constructed consisting of the same sequence amplified by the adenosine A_1 receptor primer but missing an internal 300 nucleotide fragment. Ten micrograms of a vector (TA cloning Kit, Invitrogen, Leek, The Netherlands) containing the A_1 receptor cDNA was digested by Bst E II and the fragments were separated on a preparative agarose gel. The linerarized plasmid was extracted and re-ligated overnight with T4 DNA ligase (Gibco) at 14°C. The remaining vector was cloned and sequenced by ALF and the insert used for competitive PCR. The RT mixtures were diluted 1:4 and used (4 μl) in competition assay together with known amounts (4 μl) of the deletion construct (1–1000 fg).

Amplified cDNAs were separated on ethidium bromide stained agarose gels and analyzed by gel imaging. For data analysis, the logarithm of the ratio of the density (A_1/construct) was plotted as a function of the log of the concentration of the construct and fitted by linear regression. The 0 value of the log of the ratio (A_1/construct) represents the point at which the A_1 PCR product and the construct product are present in equal amounts and therefore are also comparable before the PCR reaction (Diviacco et al. 1992; Galea and Feinstein 1992).

RESULTS

IL-6 Increases Adenosine A_1 Receptor mRNA Expression in Cultured Rat Cortical Astrocytes

Northern blot hybridisation of polyA RNA showed that 6h stimulation of rat cortical astrocyte cultures with IL-6 (100 U) causes a pronounced upregulation of adenosine A_1 receptor mRNA (Figure 1). Semi-quantitative RT-PCR experiments yielded similar results (Figure 2). This technique was utilized to study concentration-dependence and the time course of the IL-6 effect.

Figure 3A shows that already 1 U IL-6 induced a small increase in adenosine A_1 receptor mRNA expres-

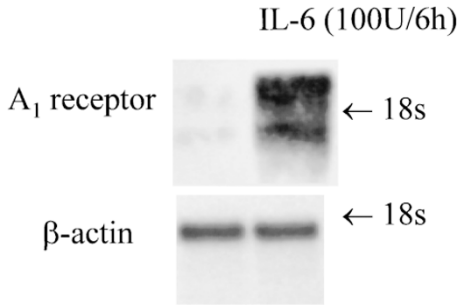

Figure 1. Northern blot analysis of polyA RNA from cultured cortical astrocytes as described in material and methods. Stimulation with IL-6 (100 U/6 h) led to an upregulation of adenosine A_1 receptor mRNA expression (upper panel); control hybridisations were performed with a cDNA probe for β-actin (lower panel). Arrows indicate the 18s RNA determined by running total RNA in the same blot. For details see Materials and Methods. Similar results were obtained in three independent experiments. IL-6 induced upregulation of adenosine A_1 receptor mRNA was 197 ± 18 %, $p \leq .01$, Student's t-test.

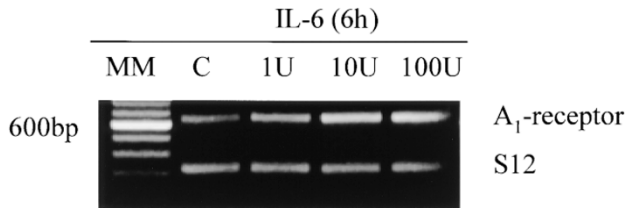

Figure 2. Effect of IL-6 stimulation (6 h) on adenosine A_1 receptor mRNA expression in cultured cortical astrocytes compared to unstimulated control (C) determined by semi-quantitative RT-PCR. This figure illustrates a typical experiment. Increase in adenosine A_1 receptor mRNA expression was determined as described in Material and Methods. Bands for adenosine A_1 receptor and S12 were 635 bp and 311 bp, respectively. MM: molecular weight marker; highlighted band is 600 bp.

sion, whereas the maximum was reached after stimulation with 10 U IL-6. No upregulation was found 1h after stimulation, but after 3 h the effect was at its maximum and remained at this plateau at least for 12 h of stimulation (Figure 3B).

Stimulation with IL-6 Increases Adenosine A_1 Receptor mRNA Expression in Organotypic Slice Cultures from Rat Cortex

It has been documented that organotypic slice cultures from neonatal rodent brain develop very similar to brain tissue *in vivo*. Therefore, these slice cultures are suitable to study the effects of long-term stimulation with drugs or hormones (for review see Gähwiler et al. 1997). Stimulation of organotypic slices with IL-6 increased the expres-

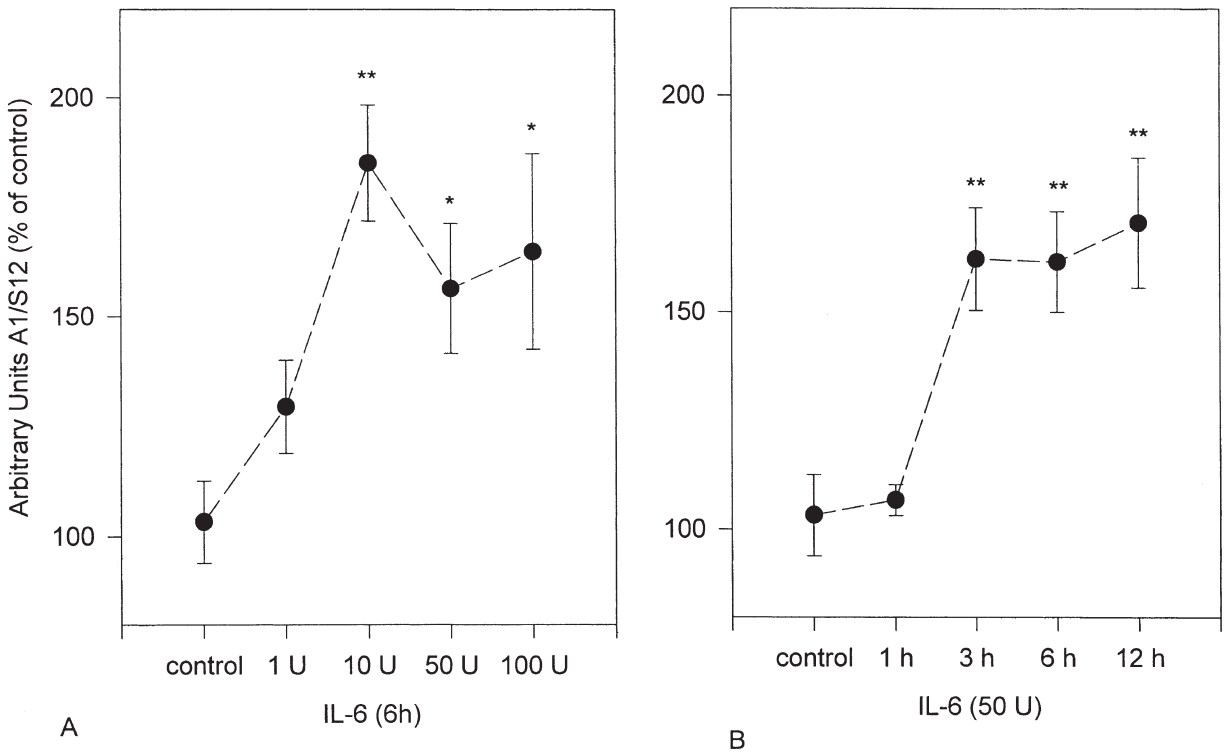

Figure 3. Concentration response curve **(A)** and time course **(B)** of IL-6 mediated upregulation of adenosine A_1 receptor mRNA expression in cultured cortical astrocytes measured by semi-quantitative RT-PCR as described in Methods. Data given are means ± SEM ($n = 4$). *, **, significantly different from unstimulated situation, $p \leq .05$ and .01, respectively; Student's t-test.

sion of adenosine A_1 receptor mRNA. The concentration- and time-dependence of this effect was comparable to that observed in astrocyte cultures: 10 U IL-6 were sufficient to induce maximal A_1-receptor mRNA upregulation (Figure 4A), A_1-receptor mRNA upregulation was significant ($p \leq .05$, Student's t-test) 3 h after stimulation (50 U IL-6) and lasted for 12 h (Figure 4B).

Stimulation with IL-6 Increases Adenosine A_1 Receptor mRNA Expression in Slices from Rat Cortex

The finding that in organotypic brain slice cultures and cultured astrocytes an IL-6 induced increase in A_1-receptor mRNA was already evident 3 h after stimulation (Figures 3B and 4B) encouraged us to ascertain potential similar effects also in freshly prepared brain slices, which are much less resistant to prolonged incubation in vitro. Indeed, 3 h incubation with 50 U/ml IL-6 increased the A_1-receptor mRNA content in the slices to 370 ± 65 % of controls ($n = 3$) (determined with semiquantitative RT-PCR; data not shown).

IL-6 (50U/6h) Induces a 2-Fold Increase in Adenosine A_1 Receptor mRNA Measured by Competitive PCR

Due to limited amounts of RNA (0.2–1 μg) it was not possible to perform Northern blots from organotypic slice cultures. Moreover, the variance of the results obtained with RT-PCR using RNA from these cultures was more pronounced as compared to cultured astrocytes (see Figures 3 and 4). Therefore, competitive PCR experiments were performed to determine the increase in mRNA expression more precisely both in astrocytes and in slice cultures. Four microliters of the cDNA sample were amplified together with a known concentration of a standard construct and the resulting fragments were separated by gel electrophoresis. Both band intensities were measured and plotted as indicated in methods and materials.

A typical experiment for cultured brain slices is shown in Figure 5. Adenosine A_1 receptor mRNA amount per preparation (10 cm Ø culture dish) in cultured astrocytes varied between 10 fg and 30 fg (unstimulated control) and between 20 fg and 70 fg (IL-6 stimulated), which revealed an upregulation of 259 ± 36% by IL-6 stimulation. In cultured cortical slices, values between 10 fg and 80 fg (unstimulated control) and 20 fg and 220 fg (IL-6 stimulated) adenosine A_1 receptor mRNA were found per preparation (three slices on a Millipore membrane), which revealed an upregulation of 230 ± 31% by IL-6 stimulation. Data are given as percent of unstimulated control (= 100%) and are means ± SD ($n = 7$), both effects are found to be significant with $p \leq .05$, Student's t-test. Thus, measurement of adeno-

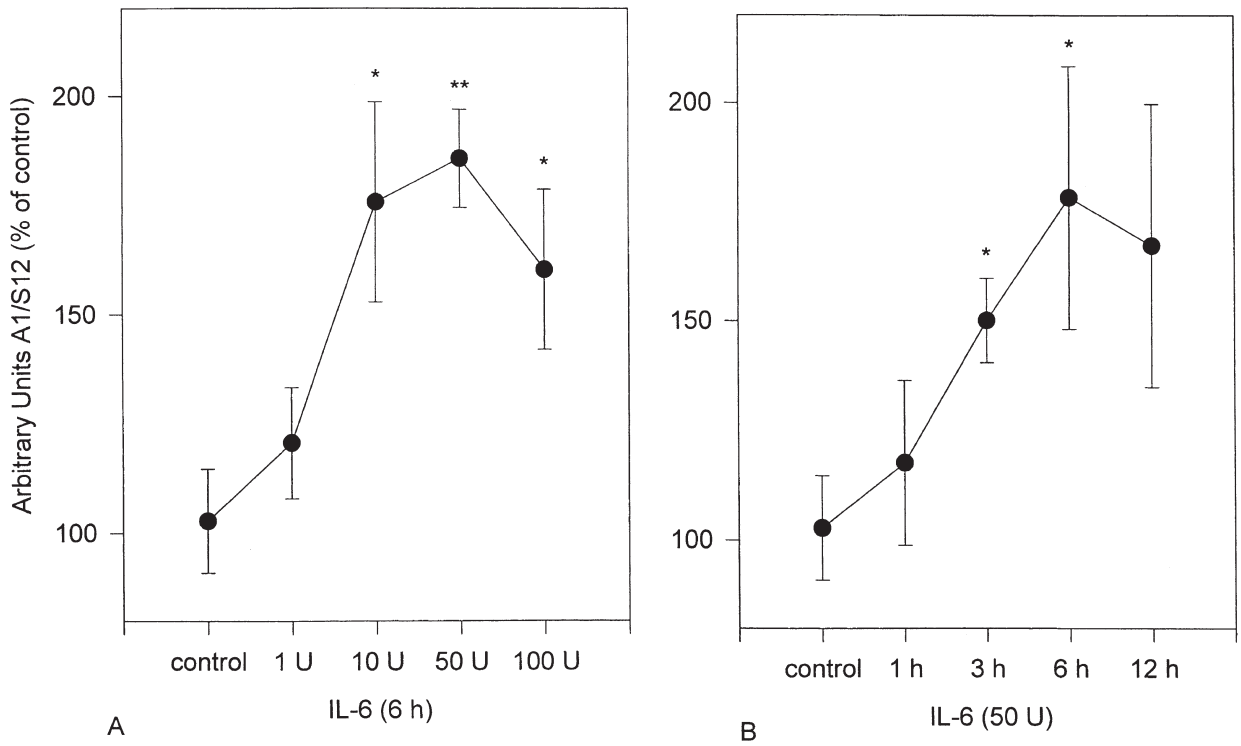

Figure 4. Concentration response curve **(A)** and time course **(B)** of IL-6 mediated upregulation of adenosine A_1 receptor mRNA expression in cultured cortical slices measured by semi-quantitative RT-PCR as described in methods. Data given are means ± SEM ($n = 5$). *, **, significantly different from unstimulated situation $p \leq .05$ and .01, respectively; Student's t-test.

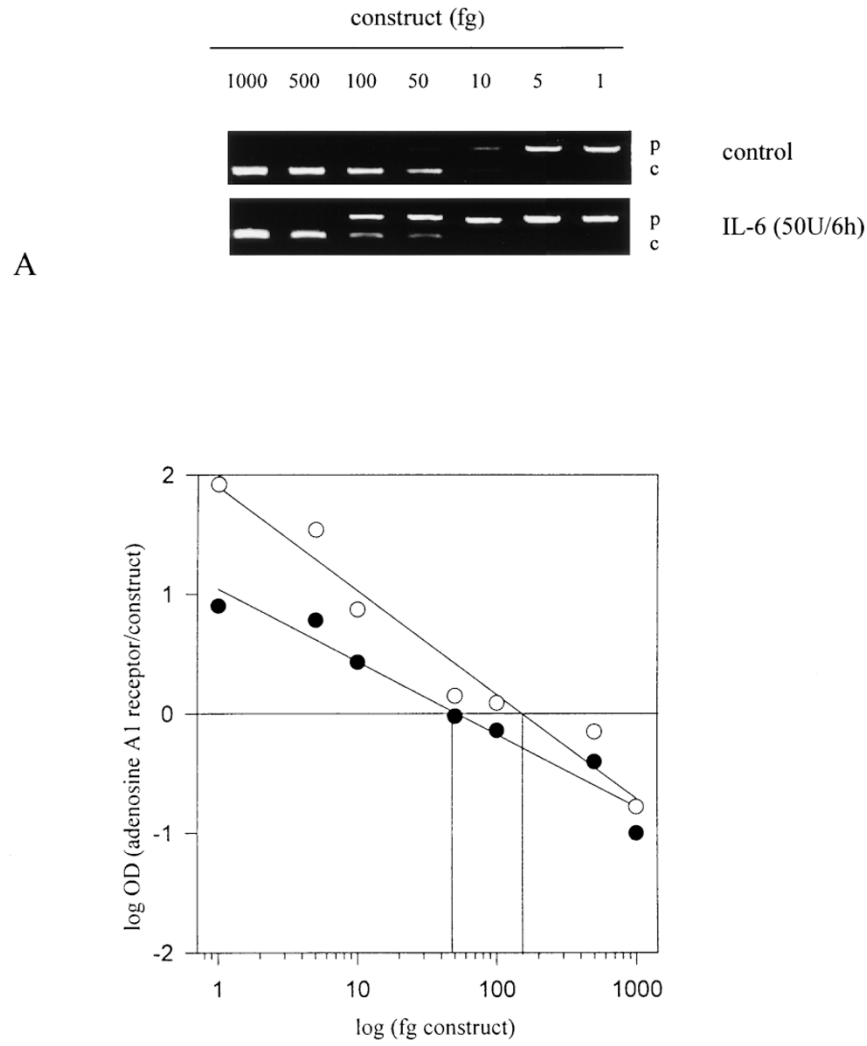

Figure 5. Upregulation of adenosine A_1 receptor mRNA expression in cultured cortical slices determined by competitive RT-PCR. **(A)** Competition between adenosine A_1 receptor PCR product (p) (635 bp) and increasing amount of a construct (c) (335 bp) produced by deletion of an internal portion of the adenosine A_1 receptor product. **(B)** Quantitative analysis of the gel presented in A. The log of the ratio of the density (adenosine A_1 receptor/construct) was plotted as a function of the log of the concentration of the construct as indicated in material and methods and fitted by linear regression analysis. IL-6 (○) treatment (50 U/6 h) induces an approximately 3-fold increase in adenosine A_1 receptor mRNA compared to untreated controls (●).

sine A_1 receptor mRNA by competitive RT-PCR confirmed the results obtained with Northern blot and semiquantitative RT-PCR and indicated in addition that IL-6 (50U/6h) increased the expression of adenosine A_1 receptor mRNA in astrocytes and brain slices by approximately a factor of 2.5 (see legend to Figure 5).

Stimulation with IL-6 Increases Adenosine A_1 Receptor Signaling

We have recently shown that adenosine A_1 receptor stimulation alone had no influence on the inositolphosphate system, whereas stimulation of A_1 receptors in astrocytes leads to a potentiation of a phenylephrine induced inositolphosphate accumulation (Biber et al. 1997). The extent of adenosine A_1 receptor-mediated potentiation of phosphoinositol signaling was found to be highly dependent on the protein expression level of these receptors (Biber et al. 1997, 1999). Therefore, we investigated the influence of IL-6 treatment on the potentiation of phenylephrine induced inositolphosphate accumulation by adenosine A_1 receptors.

IL-6 treatment had no influence on basal inositolphosphate accumulation, nor on phenylephrine induced inositolphosphate accumulation in cultured astrocytes (legend to Figure 6). However, treatment of cortical astrocyte cultures with IL-6 (100 U/16 h) significantly increased the adenosine A_1 receptor mediated potentiation of phenylephrine induced inositolphosphate accumulation as compared to control cultures without IL-6 treatment (Figure 6).

DISCUSSION

The neuromodulator adenosine acts as an endogenous neuroprotective agent in the brain (for review see Rudolphi and Schubert 1996). Some evidence also indicates a neuroprotective action of the proinflammatory cytokine IL-6 (for example, see Loddick et al. 1998; Mat-

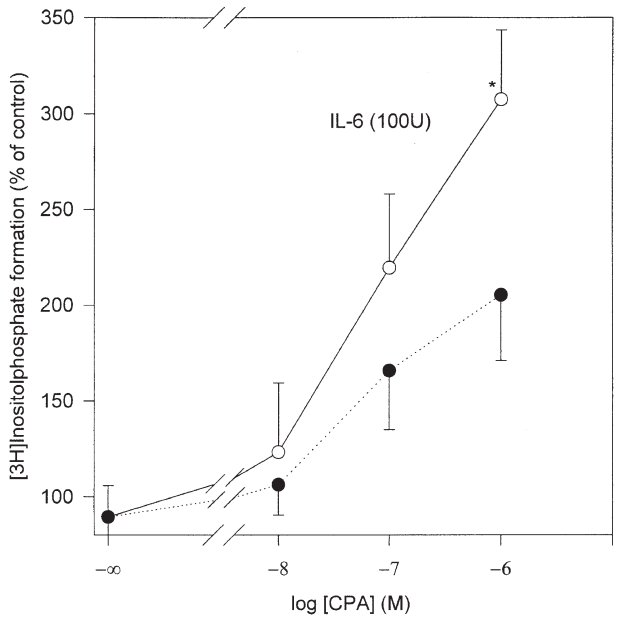

Figure 6. Effect of CPA on phenylephrine (100 μM) induced IP accumulation in cultured cortical astrocytes with (○) or without (●) IL-6 (100 U/16 h). Data are given as a percentage effect of phenylephrine-induced IP accumulation (phenylephrine (100 μM): 2350 ± 198 cpm = 100%, IL-6 (100 U/16 h) treated cultures + phenylephrin: 2384 ± 157 cpm = 100%). Basal IP levels in control cultures were 1140 ± 180 cpm and in IL-6 treated cultures 1127 ± 124 cpm. CPA alone had no influence on IP levels (CPA 1 μM; control cultures 1189 ± 167 cpm, IL-6 treated cultures 1169 ± 139 cpm). Similar results were obtained in three independent experiments. *, significantly different from untreated control $p \leq .05$; Student's t-test.

suda et al. 1996; Toulmond et al. 1992). Both compounds are synthesized and released in brain under pathological conditions such as ischemia (Loddick et al. 1998; Rudolphi and Schubert 1996).

The results of the present study together with those of previous evidence (Fiebich et al. 1996; Schwaninger et al. 1997) suggest a relationship between the neuroprotective effects of adenosine and Il-6 and provide a potential explanation for the paradoxical finding that adenosine A_1 receptors are up-regulated after sleep deprivation (Yanik and Radulovacki 1987) and ECT (Newman et al. 1984; Gleiter et al. 1989; Angelatou et al.1993; Pagonopoulou et al. 1993), despite the presence of increased adenosine concentrations under these conditions (Huston et al. 1996; Porkka-Heiskanen et al. 1997; Dragunow 1988).

We propose that increased adenosine concentrations might induce in astrocytes the synthesis and release of Il-6 (Fiebich et al. 1996; Schwaninger et al. 1997) which in turn induces upregulation of A_1 receptors in the same and/or other cells. This proposal is based on the following findings: 1) Stimulation of cultured astrocytes and cultured as well as fresh brain slices of the rat cortex with IL-6 up-regulates the expression of adenosine A_1 receptor mRNA, as determined consistently with different techniques (Northern blots, RT-PCR and competitive PCR); 2) Both the concentration dependence (maximal effect at 10 U/ml IL-6) and the time course (first effect after 3 h) of this action, as well as the extent of upregulation (measured by competitive RT-PCR; approximately 2.5 fold), were identical in astrocytes and cultured brain slices. This suggests a similar effect of IL-6 in both preparations; and 3) A pronounced effect of IL-6 on A_1 receptor mRNA expression was also observed in freshly isolated brain slices.

We have shown recently that the expression of adenosine A_1 receptor mRNA in cultured astrocytes correlates well with the expression of its receptor protein (Biber et al. 1997). Furthermore, a relationship between adenosine-mediated potentiation of inositolphosphate accumulation and A_1 receptor-expression has been described (Biber et al 1999). The increase by IL-6 of adenosine A_1 receptor mediated potentiation of PLC activity in cultured astrocytes is therefore most likely explained by an increase in adenosine A_1 receptor protein level in parallel to the observed increase in A_1 receptor mRNA. Since the effect of IL-6 on A_1-mRNA expression is observed in astrocyte cultures, these cells most probably play an important role in the similar effect observed in slice cultures and slices. It is not clear to what degree up-regulation of neuronal A_1 receptor mRNA contributes to the effect seen in slices in addition to the effect observed in astrocytes. In-situ hybridisation experiments may help to clarify this issue.

Cultured organotypic brain slices resemble brain tissue in vivo in many aspects and appear therefore to be particularly suited to study the effects of long-term stimulation with several agents (for review see Gähwiler et al. 1997). The similarity between cultured organotypic brain slices and the *in vivo* situation and the effect of IL-6 also on fresh rat brain slices makes it tempting to speculate that similar effects of IL-6 might also occur in vivo and suggests a potential relationship between the actions of adenosine and IL-6 in brain. It has been shown recently that stimulation of adenosine A_{2B} receptors in astrocytes leads to the increased synthesis of IL-6 (Fiebich et al. 1996; Schwaninger et al. 1997). Similarly, organotypic brain slice cultures and fresh rat brain slices were found to increase their content of IL-6 mRNA after stimulation with the nonselective adenosine receptor agonist 5′-N-Ethylcarboxamidoadenosine (NECA) (our unpublished results).

The adenosine A_{2B} receptor is known as the low affinity adenosine receptor which requires high concentrations of adenosine for its activation (Rudolphi et al. 1992a,b) occurring after ischemia or excitotoxicity and other pathological conditions (for review see Rudolphi and Schubert 1996). Various types of brain damage are not only associated with a large increase in extracellular adenosine but are also accompanied by an increased ex-

pression of IL-6 in the brain (Hagberg et al. 1996; Loddik et al. 1998; Schiefer et al. 1998). This implies that the stimulation of A_{2B} receptors by the high concentrations of adenosine accumulating under pathological conditions could at least partially be instrumental to the large increase in the synthesis of IL-6 in brain observed under these conditions. The results presented here furthermore suggest that this increase in IL-6 might cause beneficial effects on neuronal survival at least partially mediated via IL-6 induced up-regulation of adenosine A_1 receptors.

While the present work was in progress it was shown that stimulation of adenosine A_1 receptors induces in cultured rat astrocytes the synthesis and the release of nerve growth factor (NGF) and S-100β protein (Ciccarelli et al. 1999). These data suggest that upregulation by IL-6 of adenosine A_1 receptors in astrocytes may promote the synthesis and release of neuroprotective peptides such as NGF and S-100β protein, which may thus be instrumental to the neuroprotective effects of IL-6 and adenosine.

The relationship between adenosine and Il-6 reported in the present study may have importance beyond the neuroprotective effects of these compounds in severe brain injury. The issue of neuroprotection and neurotrophins is now recognized as an important new lead for a deeper understanding of the pathophysiology of mood disorders and the mechanisms of action of antidepressants and mood stabilizers (Duman et al. 1997; Altar 1999; Manji et al. 2000). Thus, e.g., the upregulation of adenosine A_1-receptors present after chronic treatment with carbamazepine (Daval et al. 1989) or ECT (Newman et al. 1984; Gleiter et al. 1989) might promote neuroprotection in the brain and thereby contribute to the antidepressant and prophylactic effects of these treatments.

In conclusion, we have shown that in vitro stimulation of astrocytes and brain slices with IL-6 leads to an increased expression of adenosine A_1 receptor mRNA and, in astrocytes, to an increased signalling through the phosphoinositol system. Since adenosine induces an increased synthesis of IL-6 and both IL-6 and activation of adenosine A_1 receptors exhibit neuroprotective properties, these results suggest that the neuroprotective effects of adenosine and IL-6 are intricately related and might be partially mediated via A_1-receptor upregulation. A deeper understanding of the factors that control A_1-receptor regulation in the brain could pave the ground for the development of improved therapeutic measures in patients with brain injuries and may also foster insight in the potential impact of neuroprotection in mood disorders.

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