Carbamazepine-Induced Upregulation of Adenosine A₁-Receptors in Astrocyte Cultures Affects Coupling to the Phosphoinositol Signaling Pathway

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The anticonvulsant and antibipolar drug carbamazepine (CBZ) is known to act as a specific antagonist at adenosine A₁-receptors. After a 3-week application of CBZ, A₁-receptors are upregulated in the rat brain. We have investigated the consequences of this upregulation for the A₁-receptor-mediated signal transduction in primary astrocyte cultures from different regions of the rat brain. CBZ treatment for 10 days had no effect on adenosine A₁-receptor mRNA expression in cultures with high basal A₁-receptor mRNA levels, but increased A₁-receptor mRNA in cultures exhibiting low basal A₁-receptor mRNA levels.

This upregulation of A₁-receptor mRNA was accompanied by an upregulation or induction of A₁-receptor-mediated potentiation of PLC activity, a property that was not found in these cultures before CBZ treatment. Thus, CBZ treatment for 10 days induces a new quality of adenosine A₁-receptor-mediated signal transduction in cells that express low basal A₁-receptor numbers.

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The anticonvulsant drug carbamazepine (CBZ) is known to exert antimanic and prophylactic effects in the treatment of manic-depressive disorders that are clinically equivalent to the well-known properties of lithium salts. CBZ may even have advantages as compared to lithium in the treatment and prophylaxis of schizoaffective

and rapid cycling bipolar disorders (for review see Solomon et al. 1995). Among the various pharmacological effects of CBZ, its activity as a specific antagonist at adenosine A₁ receptors (van Calker et al. 1991; Biber et al., 1996) may be particularly important, because this property could lead to alterations in the responsiveness of signal transduction systems in the brain (van Calker et al. 1998), a mechanism believed to be instrumental also to lithium's beneficial effects in affective disorders (for review see Manji et al. 1995).

Adenosine A₁- and A₂-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 signal transduction systems including phospholipase C (PLC) (Miller and Hoffman 1994; Fredholm et al. 1994). Very often, the coupling of adenosine A₁-receptors to PLCβ is synergistic with the stim-

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ulation evoked by other receptors, such as α_1 -adrenergic receptors (El-Etr et al. 1989; Biber et al. 1996, 1997), histamine H_1 -receptors (Dickenson and Hill 1994), muscarinic receptors (Biden and Browne 1993), or thyrotropin receptors (Okajima et al. 1995). There are, however, also reports suggesting direct activation of PLC by adenosine A_1 -receptors (Freund et al. 1994; Murthy and Makhoulouf 1995).

We recently reported that the stimulatory effects of adenosine A_1 -receptors on PLC signaling in primary astrocytes from different brain regions depend upon the expression level of adenosine A_1 -receptor mRNA and protein. Adenosine A_1 -receptor mediated PLC activation was only evident in cultures expressing high receptor numbers but was absent in cultures expressing low numbers of adenosine A_1 -receptors (Biber et al. 1997). We, therefore, hypothesized that upregulation of A_1 -receptors should alter their coupling to second messenger systems. Little is known about the physiological regulation of adenosine A_1 -receptor expression, but available evidence indicates an upregulation of receptor number after chronic treatment with receptor antagonists (Shi et al. 1993; Von Lubitz et al. 1994b). Thus, chronic treatment (3 weeks) with the antagonist CBZ, also results in upregulation of A_1 -receptors in the brain (Daval et al. 1989). We report here that in cultured astrocytes with low basal expression of A_1 -receptors mRNA, upregulation of receptor mRNA by CBZ treatment for 10 days induces their coupling to PLC; whereas, mRNA expression and signal transduction of adenosine A_1 -receptors in cultures with high basal mRNA levels remains unaltered.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following sources: cyclopentyladenosine (CPA), phenylephrine (PE), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and isoproterenol from Research Biochemical Inc. (RBI) and cyclic AMP-RIA Kit from Immuno Biological Laboratories (IBL), all distributed by Biotrend (Köln, Germany); Dulbecco's modified Eagle's medium (DMEM) and carbamazepine (CBZ) from Sigma (Deisenhofen, Germany); fetal calf serum from Boehringer (Mannheim, Germany); scintillation fluid (rotiszint ecoplus) from Roth (Karlsruhe, Germany); dowex anion exchanger (formate form AG 1 $\times$ 8) from Bio Rad (München, Germany); [3 H]-*myo*-inositol and [32 P]dCTP from Amersham & Buchler (Braunschweig, Germany); Bst E II from Pharmacia (Freiburg, Germany); Dynabeads from Dynal AG (Hamburg, Germany); TA cloning kit from Invitrogen (Leek, The Netherlands); random priming kit Prime IT II from Stratagen (Heidelberg, Germany); 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 (Gebicke-Härter et al. 1989). In brief, rat brains were dissected out of newborn Wistar rat pups (< 1d) under sterile conditions, and various brain regions (cortex, hippocampus, striatum, tegmentum, thalamus, and cerebellum) were isolated. Brain tissues were gently dissociated by trituration in Dulbecco's phosphate buffered saline and filtered through a cell strainer (70 μ m $\emptyset$, Falcon) into Dulbecco's modified Eagle's medium (DMEM). After two washing steps (200 $\times$ g for 10 min), cells were seeded into 24-well-dishes (Falcon) (5×10^5 cells/well). For total RNA and mRNA preparations, cells were grown in 6-well dishes (Falcon) (2×10^6 cells/dish) and standard dishes (Falcon, 10 cm $\emptyset$) (8×10^8 cells/dish), respectively. Cultures were maintained for 4 weeks in DMEM containing 10% fetal calf serum with 0.01% penicillin and 0.01% streptomycin in a humidified atmosphere (5% CO₂) at 37°C. Culture medium was changed on the 2nd day after preparation and every 6 days thereafter.

Cyclic AMP-Determination

Four-week-old cultures were washed three times with 500 μ l incubation buffer (118 mm NaCl, 4.7 mm KCl, 3 mm CaCl₂, 1.2 mm MgSO₄, 1.2 mm KH₂PO₄, 0.5 mm EDTA, 10 mm glucose, and 20 mm HEPES; pH 7.4). Cells were then stimulated at 37°C in the same buffer with the β -adrenergic receptor agonist isoproterenol (1 μ M) in the presence or absence of CPA. After 10 min, the buffer was removed, and the reaction was stopped by addition of 400 μ l ice-cold ethanol (70%). Dishes were incubated on ice for 30 min, and the supernatants were harvested. 10 μ l of each sample was dried in a speed vac centrifuge (Bachhofer, Reutlingen, Germany) and redissolved in 500 μ l sample buffer (cAMP-RIA Kit IBL). Cyclic AMP levels were determined according to the manufacturer's protocol.

Inositolphosphate Determination

Four-week-old cultures were labeled for 24 h with 1 μ Ci [3 H]-*myo*-inositol in 250 μ l of culture medium. After three washings with 500 μ l incubation buffer, 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 μ M) in the presence or absence of CPA. The reaction was stopped by addition of ice-cold TCA (100% w/v) up to a final concentration of 10% TCA. Dishes were incubated on ice for 30 min. Separation of inositolphos-

phates was performed on Dowex anion exchange columns (Formate Form AG 1 × 8) as described previously (van Calker et al. 1987). 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 and 0.1 M formic acid. Samples were mixed with 6 ml of liquid scintillation fluid and counted. Determinations were performed in triplicate.

Total RNA Extraction

Cells were lysed in guanidinium isothiocyanate/mercaptoethanol (GTC) solution (250 µl/well). Two samples were pooled to a final volume of 500 µl lysate and total RNA was extracted according to Chomczynski and Sacchi (1987).

Cloning of the cDNA Probe for Adenosine A₁ Receptors

Total RNA was reverse transcribed and PCR experiments were carried out as described previously (Biber et al. 1997). Sequences of oligonucleotide primer pairs and PCR conditions: Adenosine A₁-receptor, #55 5'<<-ATT-GCCTTGGTCTCTGTGC and #690 5'<<-CAGCTCCT-TCCCGTAGTAC, annealing temperature 59°C, 35 cycles. The PCR product was cloned using the TA cloning kit (Invitrogen) and sequenced by ALF (Pharmacia Biotech, Germany) revealing 100% homology to the known cDNA sequence for rat A₁-receptor (Mahan et al. 1991). 50 µg of the vector containing the A₁ receptor cDNA was digested by Bst E II, and the fragments were separated on a preparative agarose gel. The 300 bp fragment of the A₁-receptor cDNA was used for Northern analysis.

mRNA Extraction and Northern Analysis

Cells were lysed in 500 µl lysis/binding buffer (Dynal), 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), 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 18 S and 28 S 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 were labeled with 50 µCi [³²P]dCTP (Amersham & 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.

RESULTS

Primary astrocyte cultures from different regions of the rat brain were treated for 10 days with CBZ (50 µM). Then cultures were analyzed for (1) expression of adenosine A₁-receptor mRNA (2) adenosine A₁-receptor mediated inhibition of cyclic AMP synthesis; (3) adenosine A₁-receptor-mediated potentiation of PLC activation.

Expression of Adenosine A₁-Receptor mRNA

Adenosine A₁-receptor mRNA expression was investigated by Northern blot analysis. The results obtained for untreated cultures were similar to those previously obtained by semiquantitative RT-PCR experiments (Biber et al. 1997). Signals for adenosine A₁-receptor mRNA were found in mRNA preparations from astrocytes cultured from all brain regions investigated. Higher levels were observed in hippocampal, cortical, tegmental, and thalamic cultures (as shown in Figure 1A for hippocampal cultures); whereas, lower levels were found in cultures from whole brain, striatum, and cerebellum (as shown for cultures from cerebellum in Figure 1B). Remarkably, treatment with CBZ did not substantially alter the adenosine A₁-receptor mRNA level in cultures with high expression levels (hippocampus, cortex, thalamus, and tegmentum) (Figure 1A). However, a pronounced upregulation of adenosine A₁-receptor mRNA was observed after CBZ treatment in cultures from whole brain, striatum, and cerebellum (Figure 1B).

Inhibition of Cyclic AMP

As already reported, stimulation of adenosine A₁-receptors leads to inhibition of the isoproterenol-induced increase of cyclic AMP (van Calker et al. 1978). The maximal inhibitory effect was approximately 50% and independent of the brain region from which the astrocytes had been cultured (Biber et al. 1997). Ten-day treatment with the A₁-receptor antagonist CBZ did not alter these inhibitory effects. No significant changes of the concentration response curves after carbamazepine treatment were observed in cultures with high or with

Figure 1. Northern blot analysis of adenosine A_1 -receptor mRNA expression from primary astrocyte cultures with (+) or without (–) treatment (10 days) with CBZ (50 μ M). **(A)** Results obtained from hippocampal cultures (similar results were obtained in cultures from cortex, thalamus and tegmentum). **(B)** Results obtained from cerebellar cultures (similar results were obtained in cultures from whole brain and striatum). Control hybridizations were performed with a probe for β -actin. Results shown here are parts taken from the same northern blot. Arrows indicate 18 s and 28 s ribosomal RNA, which had been determined by running of 15 μ g total RNA in the same Northern. Three independent repetitions of the experiments gave similar results.

low A_1 mRNA expression (as shown in Figure 2 for cultures from hippocampus and cerebellum, respectively).

Potential of PLC-Activation

Astrocyte cultures established from thalamus, tegmentum, cortex, and hippocampus, expressing high levels of adenosine A_1 -receptors, exhibit adenosine A_1 -receptor-mediated potentiation of agonist-activation of PLC (Biber et al. 1997). Ten-day treatment of these cultures with CBZ did not significantly influence the signaling of adenosine A_1 -receptors to PLC (Figure 3). As shown previously, astrocyte cultures established from whole brain, cerebellum, and striatum show only moderate (whole brain) or little, if any, effect of adenosine A_1 -receptor stimulation on PLC activity (Biber et al. 1997). Ten-day treatment with CBZ, however, significantly increased the adenosine A_1 -receptor-mediated potentiation of PLC activity in cultures from whole brain (Figure 4) and induced an adenosine A_1 -receptor-mediated potentiation in cultures from striatum and cerebellum (Figure 4).

DISCUSSION

The results of the present study provide evidence that upregulation of adenosine A_1 -receptors can alter their coupling to signal transduction systems. This is concluded from the following findings.

Primary astrocyte cultures from different brain regions can be subdivided into two groups according to the expression levels of adenosine A_1 -receptor mRNA: those with high expression (cultures from hippocampus, cortex, thalamus, and tegmentum) and those with low (cerebellum, striatum) expression of A_1 -receptor

mRNA. Both determination of mRNA by Northern blot analysis (this paper) and with RT-PCR (Biber et al. 1997) revealed similar results.

Ten-day treatment with 50 μ M CBZ (which displays the same A_1 antagonistic activity as 100 nM DPCPX [Biber et al. 1996]) induced a pronounced upregulation of A_1 -receptor mRNA only in cultures with low expression (cerebellum, striatum, and whole brain). This upregulation of A_1 -receptor mRNA was accompanied by an induction (cerebellum and striatum) or upregulation (whole brain) of adenosine A_1 -receptor-mediated potentiation of PLC activation. On the other hand, CBZ treatment had no effect on mRNA expression and A_1 -receptor signaling in cultures, which already at basal conditions exhibited a high A_1 -receptor mRNA content and an A_1 -receptor-dependent PLC potentiation.

We have shown previously that in these cultures, A_1 -receptor mRNA concentration strictly correlates with receptor-binding properties. Thus, we detected in high expression cultures (hippocampus, cortex, thalamus, and tegmentum) $B_{\max}$ values of 24 ± 7 fmol/mg and in low expression cultures (striatum and cerebellum) $B_{\max}$ values of ≤ 5 fmol/mg (which was near the detection limit) (Biber et al. 1997). We verified in one experiment that, after CBZ treatment, not only adenosine A_1 -receptor mRNA is upregulated but also the receptor protein in striatal and cerebellar cultures as determined by radioligand-binding studies. Thus, CBZ treatment induced an increase of $B_{\max}$ values up to those observed in the high-affinity group. Therefore, potentiation of PLC activation by A_1 -receptors relies on the number of A_1 -receptors occupied by agonists. This is most likely explained by the bifurcating nature of G-protein signaling; that is, that the activation of *one* Gi/o protein by *one* adenosine A_1 -receptor gives rise to *two* different signal

Figure 2. Lack of effect of CBZ treatment on the inhibitory effect of CPA on the isoproterenol ($1 \mu\text{M}$)-induced accumulation of cAMP in primary astrocyte cultures from cerebellum (**A**) and hippocampus (**B**). Cultures with (Δ) or without ($\bullet$) treatment (10 days) with CBZ ($50 \mu\text{M}$). Control level of cyclic AMP in unstimulated cells: $0.5 \pm 0.4 \text{ pmol/well}$; cyclic AMP levels in isoproterenol ($1 \mu\text{M}$) stimulated cells were between $48 \pm 13 \text{ pmol/well}$ and $65 \pm 14 \text{ pmol/well}$ depending on the brain region (values are given as means of three independent experiments $\pm$ SD). Similar results were obtained from experiments performed with cultures from cortex, tegmentum, thalamus striatum, and whole brain.

transducers (α and $\beta\gamma$ subunits) with different affinities for their target enzyme. It is well known that α subunits have 10 to 100-fold higher affinities to their target enzymes than $\beta\gamma$ subunits (for review see Müller and Lohse 1995). The high affinity of α subunits for adenylyl cyclase allows an inhibition already at comparably low numbers of α subunits being activated in cultures with low A_1 -receptor mRNA and protein. In contrast, because of their low affinity to PLC, more free $\beta\gamma$ subunits are required to activate PLC, which are released in cultures with high A_1 -receptor expression (Biber et al. 1997). The results presented here support this notion, because upregulation of A_1 -receptor mRNA upregulates or even induces an A_1 -receptor mediated PLC activation but leaves unaltered the inhibition of adenylyl cyclase by A_1 -receptors.

Several other mechanisms could be envisaged to explain the CBZ-induced increase in the extent of PLC-activation; for example, changes in G-protein content, induction of specific G-protein subunits, changes in levels of PLC, or changes in inositol phospholipid availability. However, we have shown previously that G-protein and PLC contents of all cultures are comparable (measured by a direct activation of G_i/o -proteins by masto-

paran) (Biber et al. 1997). Moreover, there are no data available at the moment indicating that CBZ might have any effect on G-protein and/or PLC activity or expression or inositol phospholipid metabolism.

Adenosine A_1 -receptor up and down regulation by chronic treatment with adenosine A_1 -receptor antagonists and agonists, respectively, has been reported to modulate cerebral ischemia, seizures, spatial learning, and memory (Von Lubitz et al. 1993a,b, 1994a,b), but little is known about the physiological consequences of adenosine A_1 -receptor upregulation for their coupling to signal transduction systems. In general, there are only few reports in the literature that examine the consequences of changes in receptor expression for their signal transduction. Upregulation of receptor number may result in an increased signal transduction, as shown for the adenosine A_1 -receptor-mediated inhibition of adenylyl cyclase in chick atrial myocytes (Liang and Hirsch 1993) or the stimulation of adenylyl cyclase by vasoactive intestinal peptide receptors in leukocytes (Wiik 1991). Other reports, however, indicate that receptor upregulation did not enhance signal transduction events, as shown for bradykinin receptor 2 in human glomerular podocytes (Costenbader et al. 1997) or

Figure 3. Lack of effect of CBZ treatment on the potentiation by CPA of inositolphosphate accumulation in astrocyte cultures evoked by phenylephrine ($100 \mu\text{M}$) (=100%) from cortex (A), hippocampus (B), tegmentum (C), and thalamus (D). Cultures with (Δ) or without ($\bullet$) treatment (10 days) with carbamazepine ($50 \mu\text{M}$). Determinations were performed in triplicate and values given are means of three independent experiments $\pm$ SD.

muscarinic acetylcholin receptors in esophageal sphincter muscle (Keshavarzian et al. 1992). To our knowledge, this is the first report indicating that upregulation of a receptor induces a new quality of signal transduction.

Several treatments of affective disorders (CBZ treatment, sleep deprivation, and electroconvulsive therapy [ECT]) lead to an upregulation of adenosine A_1 -receptors in brain (for review see Durcan and Morgan 1990; Williams 1995). Our results indicate that this upregulation may lead to an increase or induction of G_i/o protein $\beta\gamma$ subunit-mediated actions. G protein $\beta\gamma$ subunits are known to have several intracellular effects (for review see Müller and Lohse 1995). Particularly, it is becoming obvious that $\beta\gamma$ subunits modify currents through several ion channels in the brain (Ma et al. 1997; Qin et al. 1997; Herlitze et al. 1996; Ikeda 1996; Wickman et al. 1994). It is, therefore, tempting to speculate that upregulation of adenosine A_1 -receptors in brain during the treatment of affective disorders may influence neuronal excitability by means of an in-

creased or induced $\beta\gamma$ subunit-mediated influence on neuronal ion fluxes and that this effect on neuronal activity may participate in the therapeutic effects of CBZ, sleep deprivation, or ECT.

In conclusion, we have provided evidence that a 10-day treatment with CBZ upregulates A_1 -receptor mRNA expression in primary astrocyte cultures from brain regions where receptor expression is low; whereas, receptor mRNA expression in cultures from brain regions where receptor expression is high were unaffected. Upregulation of adenosine A_1 -receptors had no influence on their inhibitory effect on cyclic AMP synthesis but increased or even induced adenosine A_1 -receptor-mediated potentiation of PLC activity. These results support the hypothesis that the coupling of adenosine A_1 -receptors to PLC is determined by their expression level in the cell. They provoke the question as to whether the well-known alterations in the sensitivity of receptors for neurotransmitters that are induced by various pharmacological and nonpharmacological ther-

Figure 4. Effects of CBZ treatment on the potentiation by CPA of inositolphosphate accumulation evoked by phenylephrine (100 μ M) (=100%) in astrocyte cultures from cerebellum (A), striatum (B), and whole brain (C). Cultures with (Δ) or without ($\bullet$) treatment (10 days) with carbamazepine (50 μ M). Determinations were performed in triplicate and values given are means of three independent experiments $\pm$ SD. **Significantly higher as compared to control cultures ($p < .01$, Student's t -test).

apies might also profoundly alter their signal transduction properties.

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