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Non-Selective Effects of Adenosine A_1 Receptor Ligands on Energy Metabolism and Macromolecular Biosynthesis in Cultured Central Neurons

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ABSTRACT. To investigate the effects of adenosine A_1 receptor activation on energy metabolism and RNA and protein biosynthesis in central neurons, cultured neurons from the rat forebrain were exposed for 1 hr to 72 hr to various concentrations (10 nM–100 μM) of the selective A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) or the A_1 receptor antagonist 8-cyclopentyltheophylline (CPT). At all concentrations tested, the adenosinergic compounds did not affect cell viability within 72 hr of treatment, except for CPT, which reduced viability by 19.7% when used at the concentration of 100 μM. Energy metabolism was analysed by studying the specific uptake of 2-D-[^{3}H]deoxyglucose ([^{3}H]2DG). Rates of RNA and protein biosynthesis were assessed by the measurement of [^{3}H]uridine and [^{3}H]leucine incorporation, respectively. Neuronal [^{3}H]2DG uptake was increased by 16% ($P < 0.01$) after 8 hr in the presence of 100 μM CCPA, whereas 100 μM CPT for 24 hr also increased [^{3}H]2DG uptake (8%, $P < 0.01$). At these concentrations, both ligands inhibited [^{3}H]uridine incorporation after a 3-hr treatment by 92% and 30%, respectively. CCPA never altered [^{3}H]leucine incorporation when compared to controls, and CPT significantly inhibited protein synthesis only at 10–100 μM. Additional experiments to analyse the influence of A_1 ligands on the transport of [^{3}H]2DG, [^{3}H]leucine and [^{3}H]uridine suggested that CCPA and CPT, which interact functionally with adenosine receptors by regulating cyclic AMP production in this model, are able to alter energy metabolism and RNA synthesis in central neurons in a nonspecific manner by interacting with glucose and uridine transporters. BIOCHEM PHARMACOL **55**;2: 141–149, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. rat forebrain neurons; 2-D-deoxyglucose uptake; uridine incorporation; leucine incorporation; cyclic AMP production; neuronal RNA content

Adenosine is an important modulatory compound in the mammalian central nervous system. Within the brain, adenosine levels are tightly regulated and are maintained in the nanomolar range under basal conditions. In case of metabolic stress, adenosine concentrations rise sharply and may reach up to 50 μM [1–3] to allow subsequent modulation of energy demand/supply balance [4]. Biochemical and pharmacological studies have revealed the existence of at least four types of specific receptors for adenosine [5]. Among the different subtypes, the A_1 receptors are the most widely distributed in the brain and have been mainly implicated in the neuromodulatory properties of the nucleoside. Through their coupling to G_i-proteins, adenosine A_1 receptors inhibit adenylate cyclase activity and calcium permeability, and also stimulate the formation of inositol-triphosphate and increase potassium conductance in neural cells [5, 6].

Apart from detailed investigations of second messenger pathways associated to adenosine receptors as well as extensive studies on adenosine capacity to regulate brain cell excitability [see for review 7–9], little is known about the intracellular events that mediate the physiological and biochemical neuronal responses to adenosine. In this respect, the specific effects of the nucleoside on critical mechanisms supporting neuronal functions such as energy metabolism or macromolecule biosynthesis are poorly known. Moreover, due to the existence of many cellular phenotypes in the brain which possess adenosine receptors, the interpretation of *in vivo* studies is somewhat difficult. Consequently, there is a growing interest in the development of simple model systems enabling a detailed analysis of neurochemical and molecular cell responses elicited by adenosine and related compounds, and cultured central neurons appear as a useful tool for such studies [10].

Previous investigations have shown the expression of adenosine A_1 receptors in cultured central neurons and their coupling to G proteins [11, 12]. The present work was performed to determine whether activation or blockade of adenosine A_1 receptors by specific ligands can modulate cell energy metabolism and RNA and protein biosynthesis in primary culture of central neurons.

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MATERIALS AND METHODS

Animals

Sprague–Dawley female rats (R. Janvier, Le Genest-St-Isle, France) when in the proestrous period, as shown by the observations of daily vaginal smears, were housed together with a male for 24 hr. They were then separated during their pregnancy and maintained under standard laboratory conditions on a 12:12 light/dark cycle (lights on at 0600) with food and water available *ad lib*. All animal experimentation was carried out with the highest standards of animal care, according to the *"N.I.H. Guide for Care and Use of Laboratory Animals."*

Cell Cultures

Neuronal cell cultures were obtained from 14-day-old rat embryo forebrains. Pregnant female rats were anaesthetized with halothane, and living embryos were excised by caesarian section under sterile conditions. Whole embryos were placed in culture medium previously equilibrated at 37°C, and forebrains were carefully collected. Neuronal cell suspensions were then obtained as previously described [12]. Brain tissues were dissected free of meninges and gently dispersed in a mixture of Dulbecco modified Eagle's medium (DMEM)† and Ham's F12 medium (50/50, ICN Pharmaceuticals) supplemented with 5% inactivated fetal calf serum (Gibco-BRL). After centrifugation at 700 × *g* for 10 min, the pellet was redispersed in the same medium and passed through a 46 μm-pore size nylon mesh. Density of the cell suspension was measured, and aliquots were transferred into 35 mm Petri dishes (Falcon, Becton Dickinson) precoated with poly-L-lysine in order to obtain a final density of 10^6 cells/dish. Cultures were then placed at 37°C in a humidified atmosphere of 95% air/5% CO_2. The following day, the culture medium was removed by aspiration and then replaced with a fresh hormonally defined serum-free medium consisting of the DMEM/Ham's F12 mixture enriched with human transferrin (1 mM), insulin (1 mM), putrescine (0.1 mM), progesterone (10 nM), estradiol (1 pM), sodium selenite (30 nM), and also containing fibroblast growth factor (2 ng/mL) and epidermal growth factor (10 ng/mL) (Sigma Chemical Co.). After two additional days, the culture medium was renewed with serum-free medium in the absence of growth factors.

Pharmacological Treatments with Adenosine Analogues

2-chloro-N^6-cyclopentyladenosine (CCPA) and 8-cyclopentyltheophylline (CPT, RBI) were used as selective agonist and antagonist of the A_1 adenosine receptor, respectively. Six-day-old cultures were transferred to 1.5 mL of serum-free medium containing one adenosine compound at various concentrations ranging from 10 nM to 100 μM. Cell exposure to the drug was carried out for increasing times, from 1 to 72 hr, under standardized conditions.

Cell Morphology and Viability

Cell morphology was routinely assessed by phase-contrast microscopic observations by means of a Nikon Diaphot TMD. Cell viability was assessed by a spectrophotometric method using the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma), according to Carmichael et al. [13]. Neurons were incubated at 37°C for 2 hr with MTT (500 μg/mL medium), washed twice with ice-cold buffer, and lysed in DMSO. Optical density corresponding to the converted dye was measured at 519 nm and finally expressed as a percentage of controls.

Cyclic AMP Assay

Cells were washed twice with serum-free DMEM buffered to pH 7.6 with 2.5 mM HEPES. Cultures were then incubated for 40 min at 37° in 1.5 mL of HEPES-buffered DMEM containing the phosphodiesterase inhibitor Ro 20-1724 (0.7 mM, Sigma) and adenosine deaminase (1.5 units/mL, type VII, Sigma). The adenosine A_1 receptor agonist CCPA was added 20 min after the incubation was initiated. The inhibitory effect of CCPA was measured in cells stimulated by 10 μM forskolin which was added 10 min later. The incubation was terminated by quickly removing medium. Cells were rinsed twice with ice-cold ethanol, and ethanol extracts were lyophilised for the measurement of cyclic AMP in duplicate by a competitive protein binding assay adapted from Nordstedt and Fredholm [14]. Neuronal cells remaining on the bottom of the culture dish were solubilized in 1 M NaOH and then processed for protein determination according to Bradford [15].

2-D-[^{3}H]deoxyglucose Uptake

Neuronal energy metabolism was analysed in treated and control cultures by measuring the specific uptake of 2-D-[^{3}H]deoxyglucose ([^{3}H]2DG), as previously described [16]. The culture dishes were washed twice in a HEPES-buffered Krebs–Ringer solution (125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, pH 7.4) and incubated in 2 mL of the Krebs-Ringer medium for 15 min at 37°C. The medium was then discarded and the assay was initiated by adding 1.5 mL of the buffer solution containing 1 mM [^{3}H]2DG (specific activity: 969.4 GBq/mmol, New England Nuclear). After a 30-min incubation, the radioactive medium was quickly removed and the cultures were rapidly washed three times with physiological saline. After drying, the cells were solubilized in 1 mL of 1 M NaOH, and samples were taken for scintillation counting (Beckman scintillation spectrometer, model LS-1801). Nonspecific uptake of [^{3}H]2DG was

† *Abbreviations:* CCPA, 2-chloro-N^6-cyclopentyladenosine; CPT, 8-cyclopentyltheophylline; DMEM, Dulbecco modified Eagle's medium; [^{3}H]2DG, 2-D-[^{3}H]deoxyglucose; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TCA, trichloroacetic acid.

assessed by measuring the residual radioactivity in the cultured cells by the same experimental procedure performed in the additional presence of 100 mM D-glucose.

[^{3}H]Uridine and [^{3}H]leucine Incorporation

RNA synthesis was achieved by measuring the incorporation of radiolabeled uridine during exposure to one of the tested adenosinergic compounds. The cells were incubated in serum-free medium containing [^{3}H]uridine (0.5 μCi/mL; specific activity: 0.96 TBq/mmol, New England Nuclear). At the end of exposure, [^{3}H]uridine incorporation was assessed into acid-soluble and acid-insoluble cell fractions. Cultures were washed twice with ice-cold 0.9% NaCl and treated for 20 min with 10% trichloroacetic acid (TCA) at 4°. TCA-soluble radioactivity was then collected, and the cells were washed twice with 0.5 mL of cold ethanol that was then evaporated under air stream. The TCA-insoluble fraction remaining in the culture dish was solubilized in 1 mL of 1 M NaOH. Samples were taken from both TCA-soluble and -insoluble pools for radioactivity determination, and aliquots from the TCA-insoluble fraction were used for protein measurement. L-[^{3}H]leucine (0.5 μCi/mL; specific activity: 1.924 TBq/mmol, New England Nuclear) was used for the analysis of protein synthesis, and its incorporation was assessed according to the same procedure as for uridine.

Total RNA Isolation and Quantification

In order to correlate changes in uridine incorporation following the pharmacological treatments with alterations of RNA synthesis in cultured neurons, total RNA was isolated from neuronal cells by a modification of the single-step acid guanidinium thiocyanate/phenol/chloroform extraction procedure described by Chomczynski and Sacchi [17]. The concentration of total RNA was subsequently determined in dilution samples by measuring their optical density at 260 nm.

[^{3}H]2DG, [^{3}H]uridine and [^{3}H]leucine Transport

These experiments, designed to evaluate the effects of CCPA and CPT on transport of the different tracers, were performed essentially like those for [^{3}H]2DG uptake and [^{3}H]uridine incorporation, except that the incubation time in the presence of increasing concentrations of the pharmacological compound was fixed at 6 min, a time that allows linear uptake of the tracer within the cells without significant incorporation into the acid-insoluble pool [18, 19].

Data Analysis

Raw data of [^{3}H]2DG uptake, [^{3}H]uridine and [^{3}H]leucine incorporation in treated neurons were reported per milligram of protein and compared with controls by means of global analysis of variance followed by Dunnett's test for multiple comparisons [20].

RESULTS

Cell Morphology, Viability and Purity

Six-day-old cultures grown in serum-free medium on a poly-lysine substratum showed a very high percent (more than 97%) of living cells, as demonstrated by Trypan blue exclusion, with very few (<7%) nonneuronal elements, as shown in preliminary immunocytochemical characterization using tetanus toxin binding [21], D2 protein antiserum [22], and neuron-specific enolase antiserum [23]. The cultures exhibited polygonal perikarya interconnected by a complex fiber network, whereas some distinct cellular clumps could be observed.

No morphological effects of treatments with CCPA or CPT were observed in cultured neurons after 1–72 hr of exposure at all drug concentrations tested. Cell viability, as reflected by the concentration of converted tetrazolium salt, was not altered following drug treatments, except in the presence of 100 μM CPT for 72 hr, where the neuron viability decreased significantly by 19.7% ($n = 10$) as compared to sister control cells.

Modulation of Adenylate Cyclase Activity

At the end of the 40-min incubation of the cultured cells in the presence of the phosphodiesterase inhibitor and adenosine deaminase, basal levels of cyclic AMP were found to be 2.9 ± 0.5 nM (24.5 ± 5.4 pmol/mg protein) in control neurons. Following stimulation of cyclic AMP production by the addition of 10 μM forskolin, the cells generated cyclic AMP up to 194.8 ± 15.6 nM (2350.5 ± 193.1 pmol/mg protein). As shown in Fig. 1, incubation of forskolin-stimulated neurons with increasing concentrations of CCPA led to the inhibition of cyclic AMP accumulation. Maximum reduction of cyclic AMP levels was obtained with 100 nM CCPA, with a calculated IC_{50} value of 0.9 nM.

2-D-[^{3}H]deoxyglucose Specific Uptake

Figure 2 shows that CCPA significantly increased [^{3}H]2DG specific uptake in a concentration-dependent manner between 2 and 8 hr of cell exposure to the receptor agonist. CCPA-induced increase peaked after cell treatment for 8 hr with 100 μM CCPA (+16%, $P < 0.01$), while agonist concentrations below 10 μM had no significant effects. Likewise, 100 μM CPT increased [^{3}H]2DG uptake significantly up to 24 hr of treatment (Fig. 3).

[^{3}H]uridine Incorporation

The A_1 agonist CCPA had similar inhibitory effects on [^{3}H]uridine incorporation into TCA-insoluble fraction (Fig. 4) and TCA-soluble pool (not shown). These effects

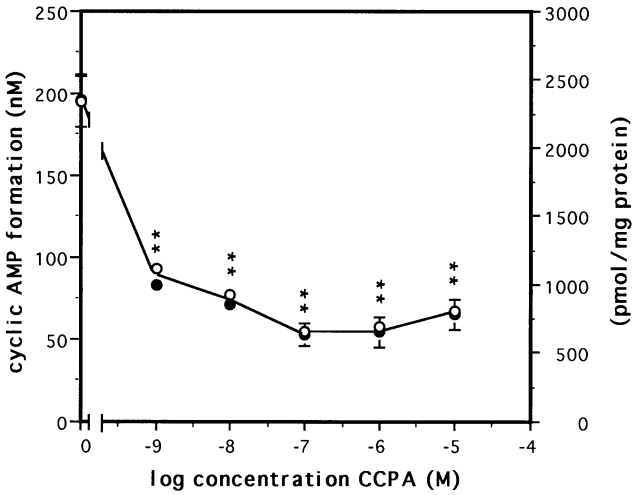

FIG. 1. Influence of CCPA on 10 μM forskolin-induced cyclic AMP formation in cultured neurons. Means ± SEM are expressed in nM (○) and in pmol/mg protein (●) and were obtained from three separate experiments conducted in duplicate in the presence of increasing concentrations of CCPA. Basal levels of cyclic AMP were 2.9 ± 0.5 nM (24.5 ± 5.4 pmol/mg protein). Statistically significant difference from controls: **$P < 0.01$ (Dunnett's test for multiple comparisons).

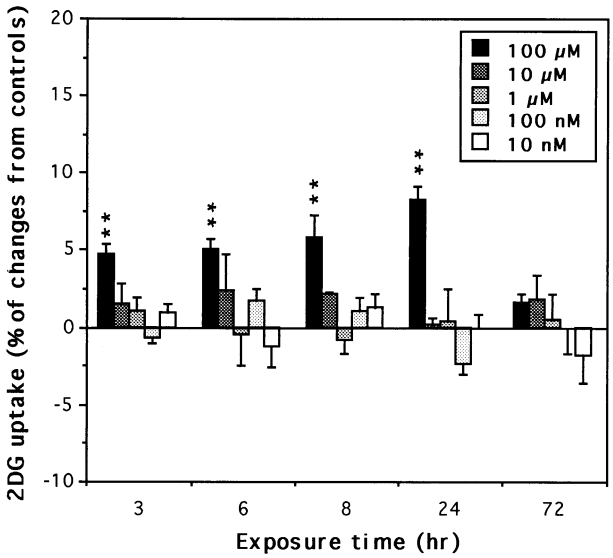

FIG. 3. Time-course of the effects of increasing concentrations of CPT on a 30-min uptake of [^{3}H]2-D-deoxyglucose by cultured neurons. Data are means ± SEM obtained from 9–25 dishes issued from at least three separate experiments. Statistically significant difference from controls: **$P < 0.01$ (Dunnett's test for multiple comparisons).

were strongly dependent on the drug concentration, [^{3}H]uridine incorporation being almost completely inhibited after a 3-h exposure to 100 μM CCPA. The inhibitory effects decreased with time of exposure to the agonist, but [^{3}H]uridine incorporation with 100 μM CCPA always remained significantly reduced as compared to controls ($P < 0.01$). [^{3}H]uridine incorporation was never significantly altered by CCPA concentrations below 1 μM, whereas 1 μM CCPA was only effective at 3 hr.

The A_1 antagonist CPT also reduced RNA synthesis (Fig. 5). Such an effect was found only at the highest concentrations used (10–100 μM). The decrease in

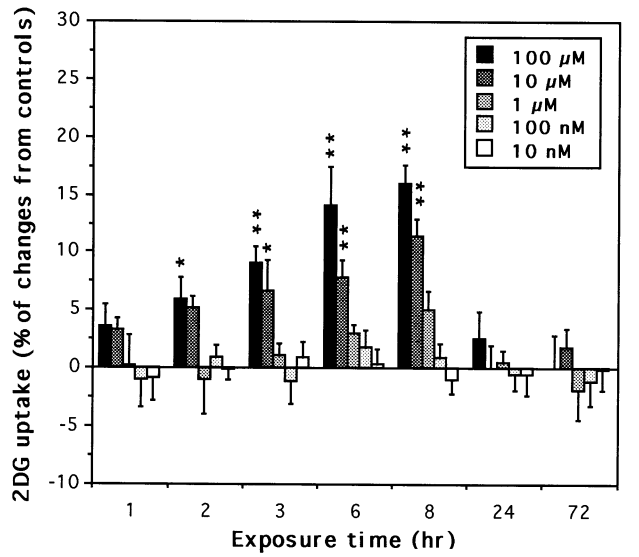

FIG. 2. Time-course of the effects of increasing concentrations of CCPA on a 30-min uptake of [^{3}H]2-D-deoxyglucose by cultured neurons. Data are means ± SEM obtained from 9–25 dishes issued from at least three separate experiments. In control neurons, [^{3}H]2-D-deoxyglucose uptake was 102.1 ± 4.7 nmol/mg protein. Statistically significant difference from controls: *$P < 0.05$ and **$P < 0.01$ (Dunnett's test for multiple comparisons).

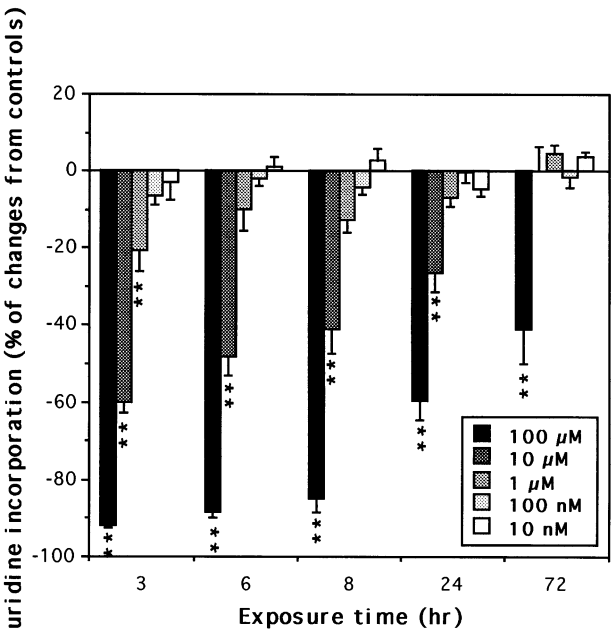

FIG. 4. Time-course of the effects of increasing concentrations of CCPA on [^{3}H]uridine incorporation into the acid-insoluble fraction of cultured neurons. Data are means ± SEM obtained from 9–40 dishes issued from at least three separate experiments. In control neurons, [^{3}H]uridine incorporation was 7744 ± 531 dpm/100 μg protein at 3 hr, and then increased with time. Statistically significant difference from controls: **$P < 0.01$ (Dunnett's test for multiple comparisons).

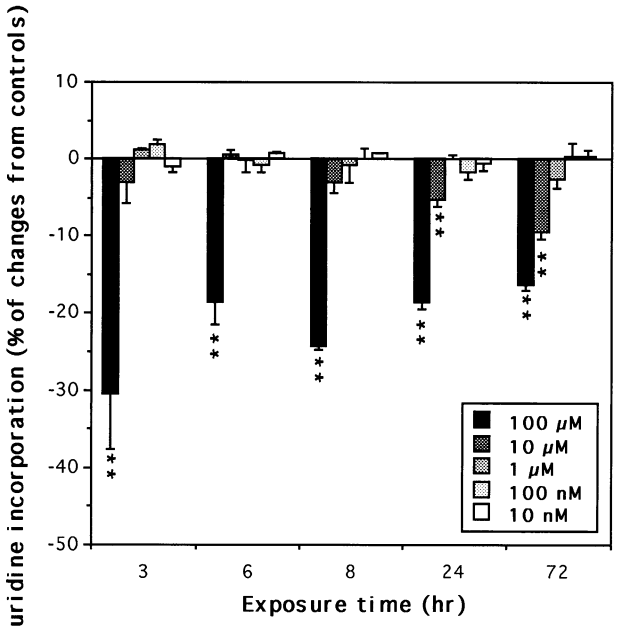

FIG. 5. Time-course of the effects of increasing concentrations of CPT on [^{3}H]uridine incorporation into the acid-insoluble fraction of cultured neurons. Data are means ± SEM obtained from 9–40 dishes issued from at least three separate experiments. Statistically significant difference from controls: **$P < 0.01$ (Dunnett's test for multiple comparisons).

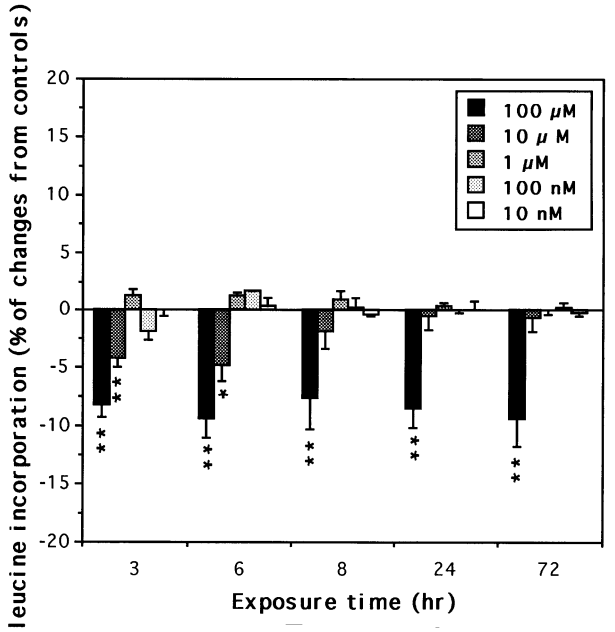

FIG. 6. Time-course of the effects of increasing concentrations of CPT on [^{3}H]leucine incorporation into the acid-insoluble fraction of cultured neurons. Data are means ± SEM obtained from 12–25 dishes issued from at least four separate experiments. In control neurons, [^{3}H]leucine incorporation was 1364 ± 99 dpm/100 μg protein at 3 hr, and then increased with time. Statistically significant difference from controls: *$P < 0.05$ and **$P < 0.01$ (Dunnett's test for multiple comparisons).

[^{3}H]uridine incorporation induced by 100 μM CPT remained basically similar with time and as for CCPA, lasted up to 72 hr of exposure. After 24 and 72 hr of treatment, incorporation rates were significantly below control values ($P < 0.01$) when cells were treated with 10 μM CPT. Uridine incorporation in the TCA-soluble fraction showed a similar profile to that observed in the TCA-insoluble fraction (data not shown).

Total RNA Concentrations

Total RNA levels were measured in four separate experiments to test the consequences of a 24-hr cell exposure to CCPA or CPT at two different concentrations (10 and 100 μM). For technical convenience, RNA amounts were quantified in pools of 6 culture dishes. In control neuronal cell cultures, RNA content was found to be 80.7 ± 4.4 μg/6 dishes.

Cell treatment with 10 μM CCPA for 24 hr significantly reduced RNA concentrations by 19.3% (65.1 ± 4.2 μg, $P < 0.05$), while 100 μM CCPA decreased total RNA by 36.4% (51.3 ± 3.9 μg, $P < 0.01$).

After 24 hr of treatment by the receptor antagonist CPT, total amounts of RNA were not affected significantly, although a 11.2% decrease was measured at the highest concentration tested.

[^{3}H]leucine Incorporation

The A_1 agonist CCPA did not significantly modify [^{3}H]leucine incorporation in either the acid-insoluble or the acid-soluble fraction, whatever the drug concentration and the duration of exposure (data not shown).

As shown in Fig. 6, the A_1 antagonist CPT (10–100 μM) significantly inhibited [^{3}H]leucine incorporation when cells were exposed to the drug for up to 6 hr. Protein synthesis remained different from controls even after 72 hr of treatment at the highest concentration used (−10%, $P < 0.01$). Concentrations of CPT below 10 μM had no effects.

Effects of A_1 Ligands on the Transport of Tracers

Influence of both ligands on [^{3}H]2DG transport is presented in Fig. 7. Transport was measured during 6-min incubation experiments in the presence of increasing concentrations of CCPA or CPT. CCPA inhibited [^{3}H]2DG transport with a calculated IC_{50} value of 180 μM, while CPT was less potent in reducing the glucose analogue transport (IC_{20} = 200 μM).

The A_1 agonist inhibited [^{3}H]uridine transport in a clear concentration-dependent manner with an IC_{50} of 4 μM (Fig. 8), while the antagonist was less effective (IC_{50} = 200 μm).

Neither receptor ligand had any effects on [^{3}H]leucine transport in cultured neurons, even when they were tested at 1 mM (data not shown).

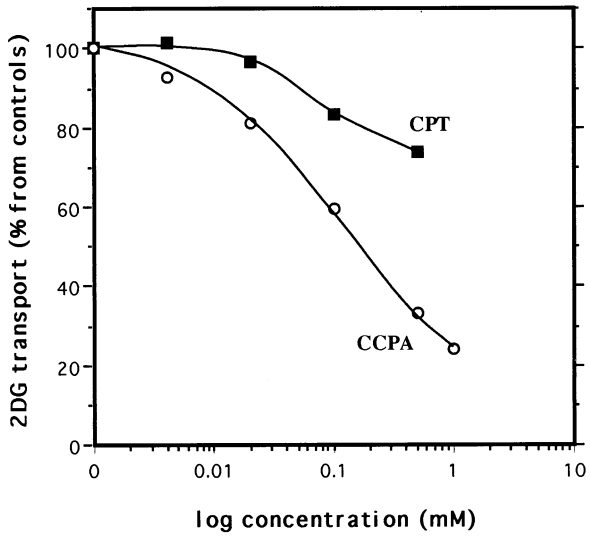

FIG. 7. Effects of CCPA (○) or CPT (■) on [^{3}H]2-D-deoxyglucose transport into cultured neurons. Specific transport was measured at the end of a 6-min incubation of the cells with the radiolabeled tracer and in the presence of increasing concentrations of the tested compound. Data were obtained from at least three separate experiments conducted in duplicate.

DISCUSSION

The present study demonstrates that whereas adenosine A_1 receptors are able to mediate the regulation of cyclic AMP production in cultured rat central neurons, alterations in neuronal energy metabolism and biosynthetic activities can be observed in the presence of their selective agonist or antagonist, and such changes mostly reflect interactions of the adenosinergic ligands with glucose and nucleoside transporters.

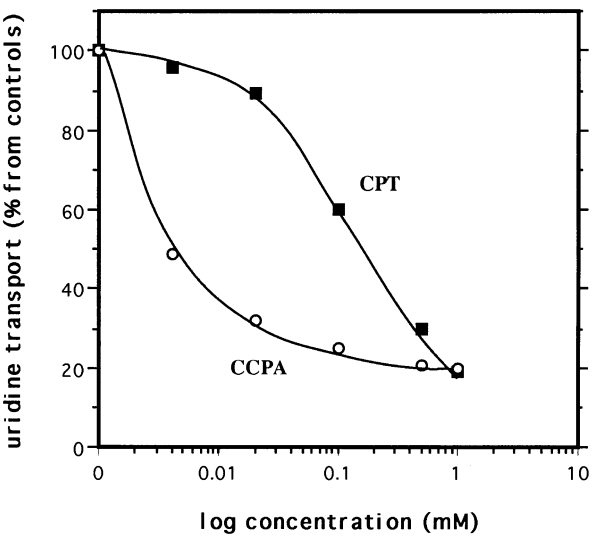

FIG. 8. Effects of CCPA (○) and CPT (■) on [^{3}H]uridine transport into cultured neurons. Specific transport was measured at the end of a 6-min incubation of the cells with the radiolabeled tracer and in the presence of increasing concentrations of the tested compound. Data were obtained from at least three separate experiments conducted in duplicate.

Evidence has been provided that adenosine is a major modulator of neuronal activity [6, 24]. The mechanisms underlying these properties are thought to be, at least partly, dependent on changes in adenylate cyclase activity, and thus in the intracellular concentration of the second messenger cyclic AMP. However, many aspects of the physiological and biochemical effects of adenosine, specifically on neurons, are still uncertain. Since cerebral adenosine receptors are largely expressed by different brain cell types [25, 26], cultured forebrain neurons were chosen to study the neurochemical consequences of A_1 receptor activation. This model exhibits a high degree of neuronal purity and viability, and neurons have been shown to express adenosine A_1 receptors which have been fully characterized [11, 12]. Using binding techniques, it has been demonstrated that A_1 receptors specifically bind CCPA with an affinity constant in the low nanomolar range, and that the receptors are tightly coupled to their G proteins [12]. Inhibition of forskolin-stimulated cyclic AMP production by CCPA with a low nanomolar IC_{50} value indicates that adenosine A_1 receptors are capable of modulating adenylate cyclase activity in cultured central neurons via their inhibitory G proteins, showing that in this culture model, adenosine A_1 receptors are functionally linked to their main effector system. Such results are consistent with previously reported studies using mixed cell cultures as well as less selective A_1 agonists [27].

Effects of CCPA and CPT on Energy Metabolism and Macromolecular Biosynthesis

Energy metabolism is a critical process for neural activity and cell survival, especially in situations when substrate availability is reduced. *In vivo* studies have reported contradictory data, with either decreases or increases in brain local metabolic rates following administration of A_1 agonists, possibly due to the inability of some adenosinergic compounds to cross the blood-brain barrier and reach their receptors [see 24]. In cultured neurons, the A_1 agonist CCPA, at concentrations of 10–100 μM, increases cellular metabolism as a function of time, but remains without effect at concentrations below 10 μM. Such results are in good accordance with those previously described both *in vivo* [28] and *in vitro* [29]. Indeed, CCPA injected intravenously in rats at low concentration (0.01 mg/kg) has no effect on glucose metabolism in forebrain areas, but is able to slightly modify local energy metabolism in other selective regions of the brain [28]. Similarly, N^6-cyclohexyladenosine, another selective adenosine A_1 receptor agonist, has no significant effect on neuronal energy metabolism when used at the concentration of 1 μM in cultured neurons maintained under physiological conditions [29].

Interestingly, the A_1 antagonist CPT displays similar basic effects to those of CCPA on cell energy metabolism, with no influence at concentrations $\leq$ 10 μM. In agreement with these observations, low concentrations of another selective adenosine A_1 receptor antagonist, 8-cyclo-

pentyl-1,3-dipropylxanthine, are also devoid of effects on cerebral metabolism in forebrain structures *in vivo* [28].

To our knowledge, there are no specific data on the effects of adenosine receptor ligands on RNA and protein biosynthesis, neither *in vitro* nor *in vivo*. Other pharmacological compounds that also modify neuronal activity, such as diazepam or phenobarbital, have been shown to alter protein and RNA synthesis in neuronal cultures [16]. In the present study, both the agonist CCPA and the antagonist CPT inhibit [^{3}H]uridine incorporation into the neurons, suggesting that these effects would not be a direct consequence of adenosine A_1 receptor activation or blockade. Such an inhibition is pronounced, rapid, dependent on the ligand concentration and leads, at least for CCPA, to parallel changes in total RNA contents in the cultured cells. Furthermore, ligand-induced alterations of uridine incorporation show similar profiles in the two cellular pools (acid-soluble and -insoluble). In contrast with the strong effects observed on [^{3}H]uridine incorporation, CCPA is without effects on [^{3}H]leucine incorporation, while 10–100 μM CPT inhibits [^{3}H]leucine incorporation only by 5–10%.

Taken together, these results indicate that in our model, A_1 receptor activation or blockade does not display significant effects on neuronal energy metabolism for agonist or antagonist concentrations, which are three orders of magnitude above their known affinity for the receptor [5, 11, 12]. They only affect metabolism at quite high concentrations, and the basically similar effects of both A_1 agonist and antagonist under these concentration conditions could not be attributed to their functional interactions with A_1 receptors. Indeed, the receptor agonist and antagonist never had opposite actions on [^{3}H]2DG uptake, as would have been expected. Similar conclusions can be drawn regarding the influence of both ligands on [^{3}H]uridine incorporation. It was thus postulated that both A_1 ligands interact with specific transporter systems.

Effects of CCPA and CPT on the Transport of Tracers

To test the above hypothesis, the duration of the incubation with the radiolabeled tracers was reduced to 6 min, a time short enough for [^{3}H]2DG, [^{3}H]uridine or [^{3}H]leucine not to be incorporated into the acid-insoluble fraction, but long enough for the radiolabeled compound to penetrate the cells. Under these conditions, the tracer accumulation in the neurons is linear with time, and only reflects transport within the cells [18, 30, 31].

The A_1 agonist CCPA inhibits [^{3}H]2DG transport with a micromolar IC_{50}, while CPT displays inhibitory properties at rather higher concentrations. Assuming that in neuronal cells, decrease in glucose availability regulates glucose transport by increasing the rate of synthesis of the transporters [31], the late increase in [^{3}H]2DG uptake observed in our study suggests a parallel increase in the number of newly synthesized glucose transporters in response to the sustained inhibition of glucose uptake by CCPA and CPT. However, this explanation needs to be confirmed by studying the actual number of glucose transporters. Previous studies have shown that when used at high concentrations, adenosine itself and theophylline, a nonspecific adenosine receptor antagonist, competitively inhibit glucose transport [19, 30].

Both CCPA and CPT at micromolar concentrations also inhibit [^{3}H]uridine transport. Other A_1 receptor ligands have already been reported as inhibitors of nucleoside transporters [30, 32–36]. It would thus appear that compounds inhibiting nucleoside transporters also have the capacity to inhibit glucose transporters. This would be the case for adenosine [19], dipyridamole [37, 38] and theophylline [30], and these observations need to be taken into consideration when interpreting pharmacological effects of adenosine A_1 receptor ligands at rather high concentrations. However, it should be noted that while both ligands do affect uridine transport into the neurons, it appears that CCPA is much more effective at lower concentrations than CPT.

The antagonist CPT, up to 1 mM, has no effects on [^{3}H]leucine transport, but does exhibit significant inhibitory effects on [^{3}H]leucine incorporation at the highest concentrations tested (10–100 μM). This inhibition cannot be explained by a direct interaction with amino acid transporters. Assuming that CPT has a relative liposolubility and a nonpolar structure [39], it is conceivable that at high concentrations, CPT could interact with membrane fluidity and thus disturb carrier function.

Physiological and Pathological Relevance

This study shows that A_1 receptor ligands have no significant effects on neuronal energy metabolism nor on RNA and protein biosynthesis when they are used at concentrations relatively close to their respective affinity-constant for the receptor ($<$100 nM). When adenosinergic compounds are used at higher concentrations ($\geq$1 μM), the inhibition of the transport of tracers suggests that while adenosine A_1 receptors may not be directly involved in the regulation of neuronal metabolism and macromolecular biosynthesis, their ligands may be potent inhibitors of the neuronal glucose and nucleoside carriers. Such results would imply that adenosine itself likely does not influence these processes under physiological conditions, since its cerebral concentrations normally remain between 30 and 300 nM [8]. However, it has been repeatedly shown that when ATP stores are highly catabolized during metabolically stressful situations such as hypoxia/ischemia or seizures, extracellular amounts of adenosine increase dramatically as the generated nucleoside escapes from the cells [1–3, 40]. Under these conditions, adenosine levels are high enough to interact with glucose and nucleoside transport and thus to affect energy metabolism and biosynthetic activities. This phenomenon may be of critical importance, especially in relation to the hypoxia/ischemia-induced expression of specific proteins encoded by putative "neuronal death

genes" [41, 42] and also to the specific changes in neuronal metabolism associated with cell damage after severe hypoxia [29]. In this respect, some of the observations described in the present study may be related to previously reported work regarding the modulatory role of adenosine in myocardial tolerance to ischemia [43, 44]. In isolated perfused rat heart preparations, adenosine infusion resulted in a dose-dependent increase in glucose uptake in the ischemic myocardium, in a manner similar to that seen with insulin. Such an A_1 receptor-mediated mechanism may participate in cardioprotection.

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References

1. Van Wylen DGL, Park TS, Rubio R and Berne RM, Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. *J Cereb Blood Flow Metab* **6:** 522–528, 1986.
2. Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S and Sandberg M, Extracellular adenosine, inosine, hypoxanthine and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. *J Neurochem* **49:** 227–231, 1987.
3. Phillis JW, Walter GA, O'Regan MH and Stair RE, Increases in cerebral cortical perfusate adenosine and inosine concentrations during hypoxia and ischemia. *J Cereb Blood Flow Metab* **7:** 679–686, 1987.
4. Bruns RF, Role of adenosine in energy supply/demand balance. *Nucleos Nucleot* **10:** 931–943, 1991.
5. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P and Williams M, Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:** 143–156, 1994.
6. Williams M, Adenosine: the prototypic neuromodulator. *Neurochem Int* **14:** 249–264, 1989.
7. Van Galen PJM, Stiles GL, Michaels G and Jacobson KA, Adenosine A_1 and A_2 receptors: structure-function relationships. *Medic Res Rev* **12:** 423–471, 1992.
8. Fredholm BB, Adenosine receptors in the central nervous system. *News in Physiol Sci* **10:** 122–128, 1995.
9. Daval JL, Nicolas F and Doriat JF, Adenosine physiology and pharmacology: How about A_2 receptors? *Pharmacol Ther* **71:** 325–335, 1996.
10. Cestelli A, Savettieri G, Salemi G and Di Liegro I, Neuronal cell cultures: a tool for investigations in developmental neurobiology. *Neurochem Res* **17:** 1163–1180, 1992.
11. Nicolas F and Daval JL, Expression of adenosine A_1 receptors in cultured neurons from fetal rat brain. *Synapse* **14:** 96–99, 1993.
12. Nicolas F, Oillet J, Koziel V and Daval JL, Characterization of adenosine receptors in a model of cultured neurons from rat forebrain. *Neurochem Res* **19:** 507–515, 1994.
13. Carmichael J, de Graff WG, Gazdar AF, Minna JD and Mitchell JB, Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* **47:** 936–942, 1987.
14. Nordstedt C and Fredholm BB, A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal Biochem* **189:** 231–234, 1990.
15. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
16. Daval JL, Pereira de Vasconcelos A and Lartaud I, Morphological and neurochemical effects of diazepam and phenobarbital on selective culture of neurons from fetal rat brain. *J Neurochem* **50:** 665–672, 1988.
17. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
18. Plagemann PGW and Roth MF, Permeation as the rate-limiting step in the phosphorylation of uridine and choline and their incorporation into macromolecules by Novikoff hepatoma cells. Competitive inhibition by phenethyl alcohol, persantin, and adenosine. *Biochemistry* **8:** 4782–4789, 1969.
19. Hara M, Matsuda Y, Hirai K, Okumura N and Nakagawa H, Characteristics of glucose transport in neuronal cells and astrocytes from rat brain in primary culture. *J Neurochem* **52:** 902–908, 1989.
20. Kirk RE, *Experimental Design: Procedures for the Behavioral Neurosciences*. Brooks-Cole, Belmont, CA, 1968.
21. Mirsky R, Wendon LB, Black P, Stolkin C and Bray D, Tetanus toxin: a cell surface marker for neurones in culture. *Brain Res* **148:** 251–259, 1978.
22. Borg J, Spitz B, Hamel G and Mark J, Selective culture of neurons from rat cerebral cortex: morphological characterization, glutamate uptake and related enzymes during maturation in various culture media. *Dev Brain Res* **18:** 37–49, 1985.
23. Marangos PJ, Schmechel D, Parma AM, Clark RL and Goodwin FK, Measurement of neuron-specific (NSE) and non-neuronal (NNE) isoenzymes of enolase in rat, monkey and human nervous tissue. *J Neurochem* **33:** 319–329, 1979.
24. Daval JL, Nehlig A and Nicolas F, Physiological and pharmalogical properties of adenosine: therapeutic implications. *Life Sci* **49:** 1435–1453, 1991.
25. Van Calker D, Muller M and Hamprecht B, Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* **33:** 999–1005, 1979.
26. Ebersolt C, Premont J, Prochiantz A, Perez M and Bockaert J, Inhibition of brain adenylate cyclase by A_1 adenosine receptors: pharmacological characteristics and location. *Brain Res* **267:** 123–129, 1983.
27. Murphy MG, Moak CM, Byczho Z and MacDonald WF, Adenosine-dependent regulation of cyclic AMP accumulation in primary cultures of rat astrocytes and neurons. *J Neurosci Res* **30:** 631–640, 1991.
28. Nehlig A, Daval JL and Boyet S, Effects of selective adenosine A_1 and A_2 receptor agonists and antagonists on local rates of energy metabolism in the rat brain. *Eur J Pharmacol* **258:** 57–66, 1994.
29. Daval JL and Nicolas F, Opposite effects of cyclohexyladenosine and theophylline on hypoxic damage in cultured neurons. *Neurosci Lett* **175:** 114–116, 1994.
30. Plagemann PGW and Sheppard JR, Competitive inhibition of the transport of nucleosides, hypoxanthine, choline and deoxyglucose by theophylline, papaverine and prostaglandins. *Biochem Biophys Res Commun* **56:** 869–875, 1974.
31. Hara M, Matsuda Y, Okumura N, Hirai K and Nakagawa H, Effect of glucose starvation on glucose transport in neuronal cells in primary culture from rat brain. *J Neurochem* **52:** 909–912, 1989.
32. Bennedetto A and Cassone A, Inhibition of uridine transport in cultured mammalian cells by theophylline. *Biochim Biophys Acta* **349:** 53–60, 1974.

33. Plagemann PGW and Wohlueter RM, Inhibition of the transport of adenosine, other nucleosides and hypoxanthine in Novikoff rat hepatoma cells by methylxanthines, papaverine, N^6-cyclohexyladenosine and N^6-phenylisopropyladenosine. *Biochem Pharmacol* **33:** 1783–1788, 1984.
34. Wu PH, Barraco RA and Phillis JW, Further studies on the inhibition of adenosine uptake into rat brain synaptosomes by adenosine derivatives and methylxanthines. *Gen Pharmacol* **15:** 251–254, 1984.
35. Lee CW and Jarvis SM, Nucleoside transport in rat cerebral-cortical synaptosomes. Evidence for two types of nucleoside transporters. *Biochem J* **249:** 557–564, 1988.
36. Balwierczak J, Krulan CM, Wang ZC, Chen J and Jeng AY, Effects of adenosine A_2 receptor agonists on nucleoside transport. *J Pharmacol Exp Ther* **251:** 279–287, 1989.
37. Scholtissek C, Studies on the uptake of nucleic acid precursors into cells in tissue culture. *Biochim Biophys Acta* **158:** 435–447, 1968.
38. Crifò C, Bozz A, Mondoví B and Strom R, Inhibition of uridine incorporation into RNA in mammalian cells by agents affecting the properties of the cell membrane: chloropromazine, dipyridamole and phenylethanol. *Biochem Pharmacol* **22:** 2511–2519, 1973.
39. Baumgold J, Nikodijevic O and Jacobson KA, Penetration of adenosine antagonists into mouse brain as determined by *ex vivo* binding. *Biochem Pharmacol* **43:** 889–894, 1992.
40. Schrader J, Wahl M, Kuschinsky W and Kreutzberg GW, Increase of adenosine content in cerebral cortex of the cat during bicuculline-induced seizure. *Pflügers Arch* **387:** 245–251, 1980.
41. Hossmann KA, Disturbances of cerebral protein synthesis and ischemic cell death. *Prog Brain Res* **96:** 161–177, 1993.
42. Dragunow M and Preston K, The role of inducible transcription factors in apoptotic nerve cell death. *Brain Res Rev* **21:** 1–28, 1995.
43. Mentzer RM, Lasley RD and Van Wylen DGL, A new role for adenosine: modulation of myocardial tolerance to ischemia. In: *Role of Adenosine and Adenine Nucleotides in the Biological System* (Eds. Imai S and Nakazawa M), pp. 547–555. Amsterdam: Elsevier, 1991.
44. Lasley RD, Bünger R and Mentzer RM, Receptor-mediated and metabolic effects of adenosine in ischemic and postischemic myocardium. In: *Adenosine and Adenine Nucleotides: from Molecular Biology to Integrative Physiology* (Eds. Belardinelli L and Pelleg A), pp. 351–360. Boston: Kluwer Academic Publishers, 1995.