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Adenosine A_1 receptor-mediated inhibition of cyclic AMP accumulation in type-2 but not type-1 rat astrocytes

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Abstract

The effects of adenosine receptor-selective ligands on [^{3}H]cyclic AMP accumulation have been investigated in type-1 and type-2 astrocyte-enriched cultures derived from neonatal rat forebrains. In type-1 astrocytes, 5′-*N*-ethylcarboxamidoadenosine (NECA) caused a concentration-dependent increase in [^{3}H]cyclic AMP accumulation (EC_{50} = 1.2 μM) which was antagonised by pretreatment with either xanthine amine congener (8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine, apparent K_d = 9 nM) or PD115,199 (*N*-[2-(dimethylamino)ethyl]-*N*-methyl-4-(1,3-dipropylxanthine)benzene sulphonamide, apparent K_d = 122 nM). In these cultures, N^6-cyclopentyladenosine (CPA), did not affect forskolin- or isoprenaline-mediated elevations of [^{3}H]cyclic AMP accumulation. These data indicate that type-1 astrocytes possess adenosine A_{2B} but not adenosine A_1 receptors coupled to adenylyl cyclase. In type-2 astrocyte-enriched cultures, 10 μM NECA caused significant elevations of [^{3}H]cyclic AMP accumulation which were similarly inhibited by either 1 μM xanthine amine congener or 10 μM PD115,199 suggesting that they were primarily due to adenosine A_{2B} receptor stimulation. However, CGS 21680 ((2-[[4-(2-carboxyethyl) phenethyl]-amino]adenosine-5′-*N*-ethylcarboxamide, 10 μM), also significantly increased [^{3}H]cyclic AMP accumulation in type-2 astrocytes suggesting the additional presence of adenosine A_{2A} receptors. Forskolin-mediated elevations of [^{3}H]cyclic AMP accumulation in type-2 astrocytes were inhibited in a concentration-dependent manner by CPA. This effect was reversed by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 μM), confirming the presence of adenosine A_1 receptors negatively coupled to adenylyl cyclase in type-2 astrocytes.

Keywords: Astrocyte, rat; cAMP accumulation; Adenosine receptor

1. Introduction

The opposing actions of adenosine on intracellular cyclic AMP accumulation in brain-derived preparations are thought to be mediated via two different, G-protein coupled, receptor subtypes; adenosine A_1 receptors which inhibit, and adenosine A_2 receptors which stimulate adenylyl cyclase activity (Van Calker et al., 1979). Adenosine A_2 receptors have now been sub-divided into 'high affinity' adenosine A_{2A} receptors and 'low affinity' adenosine A_{2B} receptors on the basis of the selective actions of the agonist, CGS 21680 (2-[[4-(2-carboxyethyl)phenethyl]-amino]adenosine-5′-*N*-ethylcarboxamide (Jarvis et al., 1989)), and the antagonist, PD115,199 (*N*-[2-(dimethylamino) ethyl]*N*-methyl-4-(1,3-dipropylxanthine)benzene sulphonamide (Bruns et al., 1987a)), at striatal membrane receptors in rat brain (for reviews see Stiles, 1992; Collis and Hourani, 1993). Support for this classification has been obtained from the molecular cloning and expression of these three receptor subtypes from various species (for review see Fredholm et al., 1994).

In previous work, we investigated the presence of adenosine receptors coupled to cyclic AMP accumulation in primary cultures of astrocytes containing mixed populations of type-1 and type-2 cells (Peakman and Hill, 1994). Using receptor subtype-selective agonists and antagonists, we confirmed the presence of adenosine A_1 and adenosine A_2 receptors coupled, respectively, negatively and positively to adenylyl cyclase. We reported that the stimulatory cyclic AMP responses observed were mediated by the adenosine A_{2B} receptor subclass and were unaffected by concurrent adenosine A_1 receptor activation. Studies have now revealed that neurotransmitter receptor expression can

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differ between the morphologically distinct astrocyte subpopulations. For example, histamine H_1 receptors coupled to phospholipase C appear to be predominantly located on type-2 astrocytes (Fukui et al., 1991; Kondou et al., 1991; Peakman and Hill, 1995) whereas histamine H_2 receptor subtypes coupled to adenylyl cyclase are primarily expressed by type-1 astrocytes (Fukui et al., 1991; Kubo et al., 1991; Peakman and Hill, 1995). In addition, using single cell imaging techniques to investigate receptor-mediated changes in intracellular free calcium ion concentration in rat astrocytes we have recently demonstrated that adenosine A_1 receptors which can couple to intracellular calcium mobilisation and extracellular calcium influx are primarily located on type-2 cells (Peakman and Hill, 1995). The imaging data together with the lack of effect of adenosine A_1 receptor stimulation on the adenosine A_{2B} receptor-mediated cyclic AMP response in mixed astrocyte cultures suggest that adenosine receptor subclasses might also be differentially distributed on the two different astrocyte types. In this paper we have therefore described the establishment and immunocytochemical characterisation of subcultures which are specifically enriched for either type-1 or type-2 astrocytes. In each of these preparations we have used receptor subtype-selective compounds to investigate the distribution of adenosine A_1 and adensoine A_2 receptors coupled to cyclic AMP accumulation.

2. Materials and methods

2.1. Cell culture

Primary cultures of astrocytes were prepared from neonatal rat forebrains and maintained as reported previously (Ruck et al., 1991; Peakman and Hill, 1994) using a modification of the methods of McCarthy and De Vellis (1980) and Ebersolt et al. (1981a,b). Enriched cultures were prepared using a modification of the method of Levison and McCarthy (1991). After 10–15 days in vitro primary cultures were shaken at 37°C for 1.5 h at 260 rpm on an orbital shaker in order to dislodge dead cells and any contaminating microglia. These were removed by replacing the growth medium. The cultures were then shaken at 350 rpm for a further 18 h to remove O-2A progenitors (the precursor cells for type-2 astrocytes) and any differentiated type-2 cells. Dislodged cells were collected through a nylon mesh (20 μm pore size) and seeded into poly-L-lysine (10 μg ml^{-1})-coated cluster dishes at approximately 8×10^4 cells ml^{-1}. Type-2 astrocyte-enriched cultures were maintained in a growth medium containing Dulbecco's modified Eagle's medium: type-1 astrocyte-conditioned medium (1:1 v/v), 20% foetal calf serum, penicillin 100 U ml^{-1} and streptomycin 100 μg ml^{-1} with daily supplements of platelet-derived growth factor (PDGF, 5 ng ml^{-1}) and basic fibroblast growth factor (bFGF, 5 ng ml^{-1}). Type-2 astrocyte-enriched cultures were used 6–10 days after subculturing. The undisturbed type-1 astrocyte monolayers remaining in the shaken flasks were allowed to recover for 2–3 days in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, then trypsinised and seeded into 24 well cluster dishes. The medium was replaced after 5 days if necessary and type-1 astrocyte-enriched cultures were used 4–10 days after subculturing. Phase contrast photographs of the subcultures were taken using a Nikon F601 camera.

2.2. Immunocytochemistry

After 18 h of shaking, dislodged cells were seeded onto glass coverslips and stained with fluorescein-conjugated antibodies for the intermediate filament protein glial fibrillary acidic protein (GFAP) or for the surface tetraganglioside, GQ (using A2B5 monoclonal antibody). Cells were fixed at room temperature with 1 ml, 3.8% formaldehyde for 15 min and when staining for GFAP they were also incubated for 15 min with 1 mg ml^{-1} glycine/0.1% Triton X 100 in PBS to enhance antibody penetration. Horse serum (10%) in phosphate buffered saline (PBS, composition mM: NaCl, 137; KCl, 2.68; KH_2PO_4, 1.47; Na_2HPO_4, 8.1) was used as a 'blocking solution' to prevent non-specific antibody binding. Monoclonal rabbit immunoglobulins to cow glial fibrillary acidic protein were diluted 1/20 in blocking solution and coverslips were incubated overnight in a humid environment at 4°C with 15 μl of the antibody solution. Monoclonal mouse immunoglobulins to mouse A2B5 antigen were diluted 1/100 in blocking solution and coverslips were incubated at room temperature for 45 min with 15 μl of the antibody solution. Control coverslips were prepared by incubating the cells with blocking solution alone. The coverslips were washed twice with 1 ml PBS and then incubated for 60 min, at room temperature, in the dark with 15 μl of fluorescein-conjugated swine anti-rabbit antisera (1/20 dilution in blocking solution) or fluorescein-conjugated rabbit anti-mouse antiserum (1/20 dilution in blocking solution) as appropriate. The coverslips were mounted in 1:1, glycerol:PBS with 1 mg ml^{-1} phenylenediamine to prevent photobleaching.

2.3. Accumulation of [^{3}H]cyclic AMP

Cyclic AMP accumulation was investigated using the method previously described (Peakman and Hill, 1994). Cells were incubated for 2 h at 37°C in 1 ml/well Hanks' Hepes (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid]) buffer (20 mM Hepes in Hanks' balanced salts solution) containing 2 mCi/well (0.08 mM) 8-[^{3}H]adenine. The labelled cell monolayers were then washed twice with Hanks' Hepes and incubated for 30 min in 1 ml/well buffer containing 100 mM rolipram, an inhibitor of the cyclic AMP selective (type IV) phosphodiesterase isoenzyme (Donaldson et al., 1988). When adenosine analogues

were used, adenosine deaminase (1.2 U ml^{-1}) was also included in the incubation medium, and where appropriate, antagonists in 10 μl buffer were also added at this stage. Agonists were added for a further 10 min period. Reactions were terminated by the addition of 50 μl concentrated hydrochloric acid which lysed the cells and precipitated proteins, and the cluster dishes were frozen overnight. [^{3}H]Cyclic AMP was isolated by sequential Dowex-alumina chromatography as described by Donaldson et al. (1988). [^{14}C]Cyclic AMP ($\sim$ 2000 dpm) was added to each sample prior to elution to correct for percentage recovery of the columns. Eluted samples were collected and the levels of [^{3}H]- and [^{14}C]cyclic AMP were determined by scintillation counting in the gel phase.

2.4. Data analysis

Agonist concentration-response curves were fitted to a logistic equation using the non-linear regression program, GraphPAD (ISI). The equation fitted was:

$$\text{response} = \frac{E_{max} \times A^n}{(EC_{50})^n + A^n}$$

where E_{max} is the maximal response, A is the agonist concentration, EC_{50} is the concentration of agonist producing half maximal stimulation, and n is the Hill coefficient.

Antagonist dissociation constants (K_d) were estimated by a modification of the null method described by Lazareno and Roberts (1987). Briefly, a concentration-response curve to NECA was generated and a concentration (C, 10 μM) of NECA was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of NECA by 50% was then determined. The NECA concentration-response curve was fitted to the logistic equation as above and a concentration of NECA (C') identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_d was then determined from the relationship:

$$C/C' = (IC_{50}/K_d) + 1$$

Statistical analysis was performed within single experiments by using an unpaired Student's t test or one way analysis of variance (ANOVA) with post-hoc Newman-Keuls. Statistical analysis was performed between multiple experiments by using two way analysis of variance (ANOVA) with post-hoc Newman-Keuls. In each experiment triplicate or quadruplicate determinations were made, however, unless otherwise stated, each value given in the text represents means $\pm$ S.E.M. of n separate experiments.

2.5. Materials

Wistar rats were obtained from the Medical School Animal Unit, University of Nottingham. DMEM was purchased from Biological Industries (Cumbernauld, UK), L-glutamine from ICN Flow Laboratories (Irvine, UK), foetal calf serum, human recombinant PDGF AA and human recombinant bFGF from Advanced Protein Products (Brierley Hill, UK) and antibiotic solution (100 $\times$) from Sigma Chemical Co. (Poole, UK). The monoclonal rabbit immunoglobulins to cow glial fibrillary acidic protein, and the fluorescent antibodies, fluorescein-conjugated swine immunoglobulins to rabbit immunoglobulins and fluorescein-conjugated rabbit immunoglobulins to mouse immunoglobulins were obtained from Dakopatt (Denmark). Monoclonal mouse immunoglobulins to mouse A2B5 antigen were supplied by Boehringer Mannheim (Germany). 8-[^{3}H]Adenine (27 Ci $mmol^{-1}$) and 8-[^{14}C]cyclic adenosine monophosphate (309 mCi $mmol^{-1}$) ammonium salt were supplied by Amersham International (Aylesbury, UK). Rolipram was a gift from Schering (Berlin, Germany) and adenosine deaminase, dissolved in glycerol, was purchased from Boehringer Mannheim (Germany). Hanks' balanced salts solution (10 $\times$) was purchased from Northumbria Biologicals (Northumberland, UK) and Hepes from Sigma Chemical Co. (Poole, UK). N^6-Cyclopentyladenosine (CPA), isoprenaline and forskolin were supplied by Sigma Chemical Co. (Poole UK). 5'-*N*-ethylcarboxamidoadenosine (NECA), (2-[[4-(2-carboxyethyl)phenethyl]-amino]-adenosine-5'-*N*-ethylcarboxamide (CGS 21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (xanthine amine congener) were all supplied by Research Biochemicals Semat (St. Albans, UK). *N*-[2-(dimethylamino)ethyl]-*N*-methyl-4-(1,3-dipropylxanthine)benzene sulphonamide (PD115,199) was a generous gift from Warner Lambert (Ann Arbor, USA).

3. Results

After 10–15 days in vitro, primary cultures of astrocytes derived from neonatal rat forebrains were comprised of a monolayer of polygonal, type-1 cells surmounted by large populations of O-2A progenitor cells and type-2 astrocytes. The O-2A precursor cells have the potential to differentiate into either oligodendrocytes or type-2 astrocytes depending on the culture medium. In the presence of 10% foetal calf serum, they develop specifically into type-2 astrocytes (Raff et al., 1983, 1984; Peakman and Hill, 1994). An 18 h period of shaking at high speed dislodged type-2 astrocytes and O-2A cells, leaving a flat monolayer of type-1 astrocytes. Cells dislodged from primary cultures were seeded into poly-L-lysine-coated cluster dishes and after two days had differentiated into the classic, stellate-shaped morphology.

3.1. Type-1 astrocytes

The mixed adenosine receptor agonist, NECA, in the presence of 100 μM rolipram and adenosine deaminase

(1.2 U ml^{-1}), caused a significant, concentration-dependent increase in the accumulation of [^{3}H]cyclic AMP in type-1 astrocyte-enriched subcultures (Fig. 1). After 10 min incubation with 0.1 mM NECA, [^{3}H]cyclic AMP levels had increased from a mean basal response of 4135 ± 305 dpm to a mean maximal response of 57 337 ± 8811 dpm (13.5 ± 2.8 fold over basal, $P < 0.01$, $n = 3$). The mean EC_{50} value calculated for this effect was 1.2 ± 0.4 μM ($n = 3$). Under the same conditions, however, the adenosine A_{2A} receptor-selective compound, CGS 21680 (Hutchison et al., 1989; Jarvis et al., 1989) had no significant effect on [^{3}H]cyclic AMP accumulation at concentrations up to 10 μM (10.5 ± 3.2% of the response to 10 μM NECA which was measured in every experiment, $n = 4$, $P > 0.05$, Fig. 1). The cyclic AMP response to 10 μM NECA, which was just maximal, was antagonised in a concentration-dependent manner by xanthine amine congener or PD115,199. The apparent K_d values calculated from individual inhibition curves for XAC and PD115,199 were 8.8 ± 3.5 nM ($n = 3$) and 121.8 ± 43.6 nM ($n = 4$) respectively.

[^{3}H]Cyclic AMP accumulation in type-1 astrocytes was significantly elevated by treatment (10 min stimulation) with either 0.1 μM isoprenaline (53.3 ± 9.3 fold over basal levels, $P < 0.01$, $n = 3$) or 1 μM forskolin (9.3 ± 4.0 fold over basal levels, $P < 0.01$, $n = 5$). Pretreatment of type-1 cells for 10 min with the adenosine A_1 receptor-selective agonist, CPA at concentrations of up to 1 μM (Lohse et al., 1988), followed by a 10 min co-incubation with CPA and 0.1 μM isoprenaline, had no significant effect on the isoprenaline response (1 μM CPA, 92.9 ± 5.4% of the control response to 0.1 μM isoprenaline alone, $P > 0.05$, $n = 3$, Fig. 2a). This was in marked contrast to results obtained using cultures containing both type-1 and type-2 astrocytes which demonstrated a significant inhibition of isoprenaline (1 μM)-mediated cyclic AMP accumulation by CPA at concentrations of 10 nM and above (Fig. 2b). Similarly, unlike in mixed astrocyte populations, CPA at concentrations up to 0.1 μM, caused no significant inhibition of the forskolin response in type-1 astrocytes (0.1 μM CPA, 87.3 ± 9.3% of the control response to 1 μM forskolin alone, $P > 0.05$, $n = 5$). At concentrations higher than 0.1 μM CPA, a significant enhancement of forskolin-mediated [^{3}H]cyclic AMP accumulation was observed (1 μM CPA, 167.0 ± 47.2% of the control response to 1 μM forskolin alone, $P < 0.01$, $n = 5$). This effect has previously been seen in cultures of mixed astrocyte populations (Woods et al., 1988; Peakman

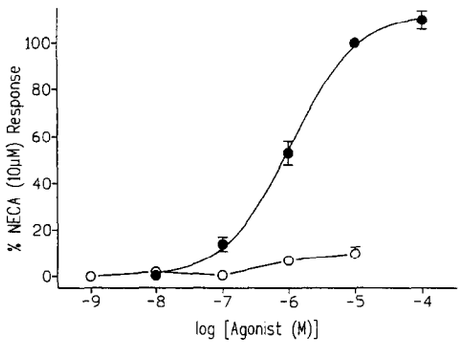

Fig. 1. Effect of NECA and CGS 21680 on accumulation of [^{3}H]cyclic AMP in type-1 astrocyte-enriched subcultures derived from rat brain. Results were obtained in the presence of rolipram 100 μM and adenosine deaminase 1.2 U ml^{-1}. Data are expressed as a percentage of the response to 10 μM NECA which was measured in every experiment. Values for NECA (●) represent means ± S.E.M. (vertical bars) from quadruplicate determinations in 3 separate experiments. Values for CGS 21680 (○) represent means ± S.E.M. (vertical bars) from triplicate determinations in four separate experiments.

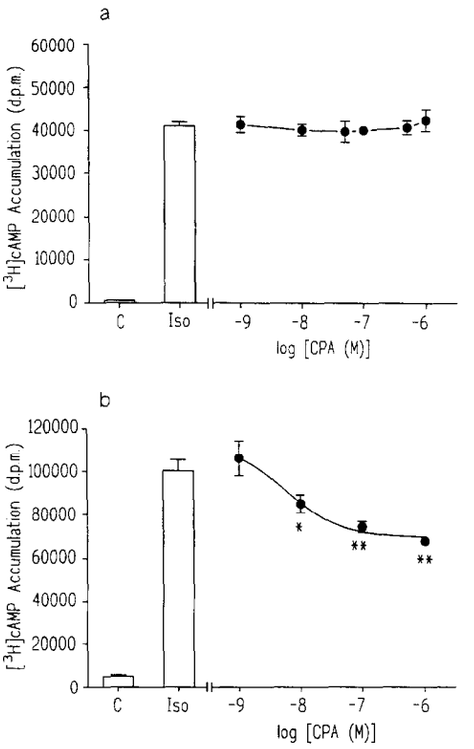

Fig. 2. Effect of N^6-cyclopentyladenosine on elevated levels of [^{3}H]cyclic AMP accumulation in type-1 astrocyte-enriched and mixed type-1 and type-2 astrocyte populations derived from rat brain. Results were obtained in the presence of rolipram 100 μM and adenosine deaminase 1.2 U ml^{-1}. The columns marked C represent the basal accumulations of [^{3}H]cyclic AMP and the columns marked Iso represents the response to 0.1 μM isoprenaline (10 min stimulation) alone. The points show the effect of increasing concentrations of N^6-cyclopentyladenosine on the isoprenaline response. Statistical significance was assessed using one way analysis of variance. a: data obtained from type-1 astrocyte-enriched cultures represent means ± S.E.M. (vertical bars) from triplicate determinations in a single experiment. There was no statistically significant inhibition of the isoprenaline response. Similar data were obtained in two other experiments. b: data obtained from primary cultures containing type-1 and type-2 astrocytes represent means ± S.E.M. (vertical bars) from quadruplicate determinations in a single experiment. Similar data were obtained in two other experiments. There was a statistically significant inhibition of the isoprenaline response at 10 nM CPA and above: * $P < 0.05$ with respect to the response to 0.1 μM isoprenaline alone; ** $P < 0.01$ with respect to the response to 0.1 μM isoprenaline alone.

and Hill, 1994), in human glioma U251 MG cells (Woods et al., 1988) and in rat brain cortical slices (Delapp and Eckols, 1992) and may result from a potentiation of the forskolin-mediated stimulation of adenylyl cyclase by the non-selective activation of adenosine A_{2B} receptor subtypes at high concentrations of CPA.

3.2. Type-2 astrocytes

After mechanical shaking of primary cultures of mixed astrocytes, dislodged cells which were seeded into 24 well cluster dishes proliferated and differentiated into a process-bearing morphology. Cell counts revealed only a $3.2 \pm 1.1\%$ ($n = 3$, 2232 cells in total) contamination by cells with the morphologies of either microglia or type-1 astrocytes. In two separate studies 24 coverslips of process-bearing cells were stained with either anti-GFAP or A2B5 antibodies. Counts of cells in a single, random field of view from each of the stained coverslips (82 cells in total) revealed that 100% of the process-bearing cells which were visible under phase contrast optics, were immunopositive for GFAP. In the same way, 88% (60 cells in total) of the process-bearing cells were immunopositive for the tetrasialoganglioside, GQ, although the staining with the A2B5 monoclonal antibody was much less intense than that seen with anti-GFAP. These data confirm that the process-bearing cells in subcultures were type-2 astrocytes and not oligodendrocytes, which do not express either GFAP or GQ (Raff et al., 1978, 1983). Unfortunately, however, the yield of these cells was extremely low. Generally, 10×75 cm^2 flasks of primary mixed astrocytes (each derived from approximately two neonates) were shaken at one time and used to generate a single 24 well cluster dish of type-2 astrocytes. Consequently, the pharmacological analysis of the responses in type-2 astrocyte-enriched preparations was somewhat restricted.

In type-2 astrocyte-enriched subcultures, NECA (10 min stimulation) caused a significant elevation of [^{3}H]cyclic AMP accumulation (10 μM NECA, 5.8 ± 2.1 fold over basal, $n = 6$, $P < 0.01$) (Fig. 3). In six separate experiments a mean basal level of 646 ± 147 dpm was elevated to a mean response level of 6020 ± 2460 dpm by 10 μM NECA. Pretreatment with 1 μM xanthine amine congener ($79.2 \pm 0.9\%$ inhibition, $P < 0.01$, $n = 3$) or 10 μM PD115,199 ($55.5 \pm 12.8\%$ inhibition, $P < 0.01$, $n = 3$), caused significant, sub-maximal inhibitions of the cyclic AMP response to 10 μM NECA (10 min stimulation) which were similar in magnitude in each case (Fig. 3). These antagonists were used at ten-fold different concentrations, since at the adenosine A_{2B} receptor sites previously characterised in mixed astrocyte populations and on type-1 astrocytes (Peakman and Hill, 1994), the affinity of PD115,199 was approximately one order of magnitude lower than that of xanthine amine congener. Interestingly, the adenosine A_{2A} receptor-selective agonist, CGS 21680 significantly increased the accumulation of [^{3}H]cyclic AMP in type-2 astrocytes. In six experiments, the mean response to 10 μM CGS 21680 was 2.8 ± 0.6 fold over basal ($P < 0.01$) and $33.4 \pm 15.2\%$ of the response to 10 μM NECA which was measured in every experiment (Fig. 3).

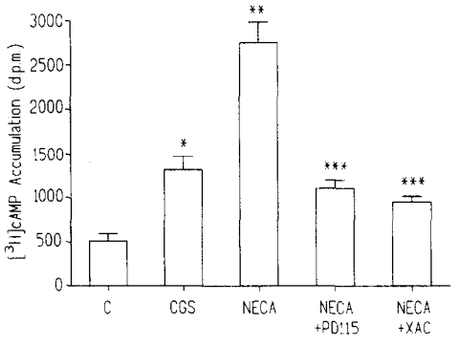

Fig. 3. Effects of adenosine receptor selective compounds on [^{3}H]cyclic AMP accumulation in type-2 astrocyte-enriched subcultures derived from rat brain. All results were obtained in the presence of rolipram 100 μM and adenosine deaminase 1.2 U ml^{-1}. Where appropriate, XAC or PD115,199 were added to the incubation medium 30 min prior to NECA addition. Receptor agonists were incubated with the cells for 10 min. Abbreviations: C, control (basal levels of [^{3}H]cyclic AMP accumulation); CGS, 10 μM CGS 21680; NECA, 10 μM NECA; PD115, 10 μM PD115,199; XAC, 1 μM xanthine amine congener. Values represent the means ± S.E.M. (vertical bars) of [^{3}H]cyclic AMP accumulations from triplicate determinations in a single experiment. Similar results were obtained in five (CGS 21680 and NECA) or two (PD115,199 and XAC) other experiments. Statistical significance was assessed using one way analysis of variance: * $P < 0.05$ with respect to basal levels; ** $P < 0.01$ with respect to basal levels; *** $P < 0.01$ with respect to NECA response.

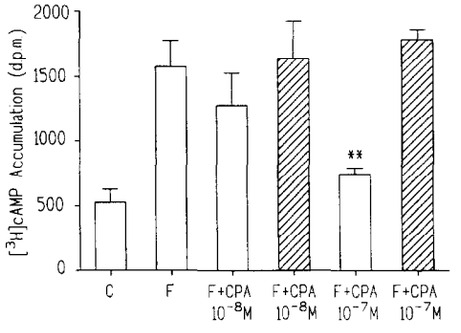

Fig. 4. Effect of adenosine A_1 receptor selective compounds on the forskolin-mediated accumulation of [^{3}H]cyclic AMP in type-2 astrocyte-enriched subcultures derived from rat brain. All results were obtained in the presence of rolipram 100 μM and adenosine deaminase 1.2 U ml^{-1}. Hatched columns represent the additional presence of DPCPX 0.1 μM. Where appropriate, DPCPX was added to the incubation medium 30 min before CPA addition which occurred 10 min before forskolin addition. Co-incubations with all compounds lasted for a further 10 min. Abbreviations: C, control (basal levels of [^{3}H]cyclic AMP accumulation); F, forskolin (1 μM); CPA, N^6-cyclopentyladenosine. Values represent the means ± S.E.M. (vertical bars) of [^{3}H]cyclic AMP accumulations from triplicate determinations in a single experiment. Similar results were obtained in five (F+CPA 10^{-7} M) or two (F+CPA 10^{-8} M) other experiments. Statistical significance was assessed using one way analysis of variance: ** $P < 0.01$ with respect to the response to forskolin (1 μM) alone.

Forskolin (1 μM, 10 min stimulation) significantly elevated [^{3}H]cyclic AMP accumulation in type-2 astrocytes (5.7 ± 1.1 fold over basal, $P < 0.01$, $n = 6$) (Fig. 4). In order to investigate the presence of adenosine A_1 receptors, these cells were pretreated with CPA for 10 min before forskolin (1 μM) addition and then co-incubated with both compounds for a further 10 min. In six experiments, a 62.7 ± 12.2% inhibition ($n = 6$, $P < 0.01$) of the forskolin response was observed after treatment with 0.1 μM CPA. The inhibitory response to 0.1 μM CPA was antagonised by the adenosine A_1 receptor-selective antagonist, DPCPX (0.1 μM, 30 min pretreatment followed by 10 min co-incubation with CPA alone and 10 min co-incubation with both CPA and forskolin). In the presence of 0.1 μM DPCPX, 0.1 μM CPA caused only a 9.3 ± 15.6% inhibition of the response to 1 μM forskolin ($P > 0.05$, $n = 6$).

4. Discussion

Secondary cultures enriched for the different astrocyte subtypes were prepared by the mechanical removal of O-2A progenitor cells and type-2 astrocytes from primary mixed astrocyte cultures. The resulting subcultures of process-bearing cells were characterised by immunostaining for GFAP and the tetrasialoganglioside GQ to ensure that the alteration in growth conditions had not induced the progenitor cells to differentiate into oligodendrocytes (Raff et al., 1983, 1984).

4.1. Type-1 astrocytes

NECA significantly elevated [^{3}H]cyclic AMP accumulation in type-1 astrocyte-enriched subcultures in a concentration-dependent manner and with a mean EC_{50} value (1.2 ± 0.4 μM; $n = 3$) which was in close agreement with the value of 1.3 μM, previously calculated using primary cultures of mixed astrocyte populations (Murphy et al., 1991; Peakman and Hill, 1994). In addition, both the actual size of the response and the fold stimulation produced by NECA were similar in mixed (10 μM NECA, 64 000 ± 9000 dpm, 9.7 ± 1.6 fold over basal, $n = 8$ (Peakman and Hill, 1994)) and type-1-enriched (10 μM NECA, 52 000 ± 18 000 d.p.m., 12.4 ± 2.6 fold over basal, $n = 3$) cultures. These data indicate that type-1 astrocytes possess adenosine A_2 receptors coupled to cyclic AMP accumulation which are likely to be of the adenosine A_{2B} subtype previously reported in mixed astrocyte cultures. This is further supported by the potency of NECA relative to CGS 21680, which had no apparent effect at concentrations up to 10 μM in these studies. In contrast, at adenosine A_{2A} receptors in rat striatal membranes, CGS 21680 exhibits a high affinity ($K_d = 16$ nM) similar to that of NECA ($K_d = 10$ nM) (Bruns et al., 1986).

Xanthine amine congener has a seven-fold selectivity for adenosine A_1 receptors in binding studies using rat brain membranes ($K_d = 4$ nM (Lohse et al., 1987)) but does not discriminate between adenosine A_2 receptor subtypes. In type-1 astrocytes xanthine amine congener antagonised the [^{3}H]cyclic AMP response to 10 μM NECA with a K_d value of 8.8 ± 3.5 nM ($n = 3$) which correlated well with that of 11.5 nM calculated for mixed astrocyte cultures (Peakman and Hill, 1994) and with values reported at adenosine A_{2A} receptors in rat striatal membranes (Lohse et al., 1987) and adenosine A_{2B} receptors in the guinea-pig cerebral cortex and aorta (Alexander et al., 1994). PD115,199 has previously been used to discriminate between adenosine A_2 receptor subtypes in guinea-pig tissues (Alexander et al., 1994) and mixed astrocyte populations (Peakman and Hill, 1994). It exhibits a similar, high affinity at both adenosine A_1 ($K_d = 14$ nM) and adenosine A_{2A} receptors ($K_d = 16$ nM) in rat whole brain and striatal membranes respectively (Bruns et al., 1987b), which is comparable to the affinity of xanthine amine congener at adenosine A_2 receptors. In this study, however, PD115,199 displayed a 14-fold lower affinity than xanthine amine congener. Its K_d value of 121.8 ± 43.6 nM ($n = 4$) was eight-fold higher than has previously been reported at adenosine A_{2A} receptor sites in rat striatal membranes (16 nM (Bruns et al., 1987b) but agreed with the K_d of 134 nM calculated in mixed astrocyte cultures (Peakman and Hill, 1994) and with values recorded at adenosine A_{2B} receptor sites in the guinea-pig aorta and cerebral cortex (Alexander et al., 1994). Taken together these data imply the presence of adenosine A_{2B} receptors coupled to cyclic AMP accumulation on type-1 astrocytes.

The forskolin (1 μM)-mediated elevation of [^{3}H]cyclic AMP accumulation in type-1 astrocyte-enriched cultures was of a similar order of magnitude (30 000 ± 6000, 9.3 ± 4.0 fold over basal, $n = 5$) to the elevation previously observed in mixed astrocyte cultures (90 000 ± 9000 dpm, 20.9 ± 2.0 fold over basal, $n = 21$ (Peakman and Hill, 1994)). The lack of a CPA-mediated inhibition of either forskolin- or isoprenaline-stimulated [^{3}H]cyclic AMP responses, however, indicated that type-1 cells do not possess adenosine A_1 receptors negatively coupled via a G_i-protein to adenylyl cyclase. These data do not exclude the possibilities that adenosine A_1 receptors might instead couple to G_o-proteins (Offermanns and Schultz, 1994) in type-1 astrocytes or be unable to couple to adenylyl cyclase due to a lack of signal transducing G_i-proteins. The latter suggestion is unlikely since a presumably G_i-protein-mediated inhibition of isoprenaline (1 μM)-stimulated [^{3}H]cyclic AMP accumulation has been recorded in type-1 astrocytes after α_2-adrenoceptor (Ruck et al., 1991) or metabotropic glutamate receptor (Baba et al., 1993) activation. Consistent with the former suggestion, our recent work has demonstrated CPA (10 μM)-mediated elevations of intracellular free calcium ion concentration in 23% of single type-1 astrocytes present in mixed astrocyte cultures (Peakman and Hill, 1995). It also remains possible

that the presence of type-1 astrocytes possessing adenosine A_1 receptors coupled to adenylyl cyclase varies with rat brain region, as appears to be the case for numerous other cell characteristics (see Wilkin et al., 1990), and that in these cultures (derived from the whole rat forebrain) the number of receptors coupled to this second messenger system is too low to produce a recordable functional response.

4.2. Type-2 astrocytes

Due to the low yield of process-bearing cells obtained after mechanical shaking of mixed astrocyte cultures, studies using type-2 astrocyte-enriched populations were limited to investigations of the effects of adenosine receptor-selective agonists and antagonists used at specific concentrations only. NECA (10 μM) significantly increased the accumulation of [^{3}H]cyclic AMP in type-2 astrocytes indicating the presence of adenosine A_2 receptors on these cells. The uptake of [^{3}H]adenine by these cultures and the fold stimulation of the NECA response were not significantly different from those observed in type-1 astrocyte-enriched cultures (10 μM NECA, 12.4 ± 2.6 fold over basal, $n = 3$) or in mixed astrocytes (10 μM NECA, 9.7 ± 1.6 fold over basal, $n = 8$, Peakman and Hill, 1994). There was, however, a significantly lower ($0.40 \pm 0.12\%$, $n = 6$) percentage conversion of [^{3}H]adenine to [^{3}H]cyclic AMP in type-2 astrocytes in the presence of 10 μM NECA, than in type-1 astrocytes ($4.51 \pm 0.16\%$, $n = 11$) ($P < 0.001$, unpaired Student's t test) which was reflected in the size of the NECA-mediated response in each preparation. These observations may be due to the presence of adenosine A_1 receptors on type-2 astrocytes which would be simultaneously stimulated by treatment with NECA, causing an inhibition of cyclic AMP accumulation. Alternatively, they may reflect differences in adenylyl cyclase activities or G-protein coupling efficiencies in type-1 and type-2 astrocytes.

The antagonism of NECA 10 μM-mediated [^{3}H]cyclic AMP accumulation in type-2 astrocytes by 1 μM xanthine amine congener was sub-maximal and similar to (or slightly greater than) that mediated by a ten-fold higher concentration of PD115,199. These data are consistent with antagonist activity at adenosine A_{2B} receptors. It therefore appears that a major component of the NECA-mediated elevation of [^{3}H]cyclic AMP accumulation in type-2 astrocytes is mediated via adenosine A_{2B} receptors. The additional presence of an adenosine A_{2A} receptor population on these cells is, however, suggested by the effect of CGS 21680. Treatment (10 min stimulation) with 10 μM CGS 21680, a selective adenosine A_{2A} receptor agonist (K_d = 15.5 nM (Jarvis et al., 1989; see also Lupica et al., 1990), caused a significant elevation in the accumulation of [^{3}H]cyclic AMP in type-2 astrocyte-enriched cultures. The size of this response was $33.4 \pm 15.2\%$ of that to 10 μM NECA (measured in every experiment) which was notably larger than the relative stimulations of 8% and 11% observed in mixed (Peakman and Hill, 1994) and type-1-enriched astrocyte populations respectively. Taken together these data would suggest that type-2 astrocytes, unlike type-1 cells, appear to possess both adenosine A_{2B} and adenosine A_{2A} receptor populations, the former of which accounts for the major proportion of the cyclic AMP response to NECA.

Treatment with 1 μM forskolin directly stimulated adenylyl cyclase activity in type-2 astrocytes. Inhibition of the forskolin-mediated response by the adenosine A_1 receptor-selective agonist, CPA (0.1 μM) and the antagonism of this effect by DPCPX (0.1 μM) implied the presence of adenosine A_1 receptors negatively coupled to adenylyl cyclase on these cells. The percentage conversion of [^{3}H]adenine to [^{3}H]cyclic AMP mediated by forskolin was significantly lower ($0.33 \pm 0.04\%$, $n = 6$) in these cultures than in type-1 astrocyte-enriched preparations ($2.81 \pm 0.29\%$, $n = 8$). This was reflected by a marked contrast in the magnitudes of forskolin-mediated responses and indicated that cyclic AMP accumulation in type-1 and type-2 astrocytes differed at some post-G-protein site, for example at the level of adenylyl cyclase. Since, we have recently shown that adenosine A_1 receptors which can augment histamine H_1 receptor-mediated intracellular calcium responses are predominantly located on type-2 astrocytes present in mixed astroglial cultures (Peakman and Hill, 1995), the presence of functional adenosine A_1 receptors negatively coupled to adenylyl cyclase in purified cultures of type-2 astrocytes is consistent with our previous observations.

4.3. Functional significance

Although it is clear that astrocytes have key roles in the regulation of the brain microenvironment (Murphy, 1993), there is still little information available regarding the functional differences between type-1 and type-2 astrocyte subtypes. Indeed, there is still much controversy regarding the correlate of type-2 astrocytes in vivo. It has been suggested that subpopulations of the 'protoplasmic' astrocytes which have been described in vivo may arise from O-2A progenitor cells (see Murphy, 1993). The demonstration of a functional significance of type-2 astrocytes may provide both an incentive and the means to identify these cells within the brain.

There is now growing evidence for receptor heterogeneity in cultured astrocyte preparations (Wilkin et al., 1990; McCarthy and Salm, 1991; Peakman and Hill, 1995). This differential distribution may allow astrocytes in different brain regions in vivo to interact specifically with neurones in their immediate environment. The distribution of adenosine receptor subtypes on glial cells may be important following insults such as stroke, ischaemia and brain trauma which are characterised by a massive release of excitatory amino acids leading to neuronal hyperactivation and death

(Ransom et al., 1990; Rudolphi et al., 1992). Under these conditions, extracellular adenosine levels also increase dramatically and the ability of adenosine to increase compound action potential recovery in the optic nerve following anoxia is indicative of its neuroprotective effect (Fern et al., 1994). Hence, the differential location of adenosine receptor subtypes on type-1 and type-2 astrocytes in vivo is most interesting since it may enable the selective activation of second messenger systems in specific astrocyte populations in order to mediate the protective effects of adenosine. On the other hand, a full characterisation of adenosine receptors present on cells of the O-2A lineage following differentiation into oligodendrocytes still remains to be undertaken.

In conclusion, the data from this study indicate that adenosine receptors coupled to cyclic AMP accumulation are differentially distributed on the two different astrocyte morphologies. Type-1 astrocytes appear to possess positively coupled adenosine A_{2B} but not negatively coupled adenosine A_1 receptor subtypes. Type-2 astrocytes, on the other hand, appear to possess both adenosine A_1 and adenosine A_2 receptors linked to adenylyl cyclase. Moreover, the adenosine A_2 receptor-mediated elevation of cyclic AMP accumulation in type-2 astrocytes appears, from preliminary data, to be comprised of both adenosine A_{2A} and adenosine A_{2B} receptor-mediated components.

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