Adenosine A₁ receptor-mediated changes in basal and histamine-stimulated levels of intracellular calcium in primary rat astrocytes

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1 The effects of adenosine A₁ receptor stimulation on basal and histamine-stimulated levels of intracellular free calcium ion concentration ($[Ca^{2+}]_i$) have been investigated in primary astrocyte cultures derived from neonatal rat forebrains.

2 Histamine (0.1 μ M–1 mM) caused rapid, concentration-dependent increases in $[Ca^{2+}]_i$ over basal levels in single type-2 astrocytes in the presence of extracellular calcium. A maximum mean increase of $1,468 \pm 94$ nM over basal levels was recorded in 90% of type-2 cells treated with 1 mM histamine ($n=49$). The percentage of type-2 cells exhibiting calcium increases in response to histamine appeared to vary in a concentration-dependent manner. However, the application of 1 mM histamine to type-1 astrocytes had less effect, eliciting a mean increase in $[Ca^{2+}]_i$ of 805 ± 197 nM over basal levels in only 30% of the cells observed ($n=24$).

3 In the presence of extracellular calcium, the A₁ receptor-selective agonist, N⁶-cyclopentyladenosine (CPA, 10 μ M), caused a maximum mean increase in $[Ca^{2+}]_i$ of $1,110 \pm 181$ nM over basal levels in 30% of type-2 astrocytes observed ($n=53$). The size of this response was concentration-dependent; however, the percentage of type-2 cells exhibiting calcium increases in response to CPA did not appear to vary in a concentration-dependent manner. A mean calcium increase of 605 ± 89 nM over basal levels was also recorded in 23% of type-1 astrocytes treated with 10 μ M CPA ($n=30$).

4 In the absence of extracellular calcium, in medium containing 0.1 mM EGTA, a mean increase in $[Ca^{2+}]_i$ of 504 ± 67 nM over basal levels was recorded in 41% of type-2 astrocytes observed ($n=41$) after stimulation with 1 μ M CPA. However, in the presence of extracellular calcium, pretreatment with the A₁ receptor-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine, for 5–10 min before stimulation with 1 μ M CPA, completely antagonized the response in 100% of the cells observed.

5 In type-2 astrocytes, prestimulation with 10 nM CPA significantly increased the size of the calcium response produced by 0.1 μ M histamine and the percentage of responding cells. Treatment with 0.1 μ M histamine alone caused a mean calcium increase of 268 ± 34 nM in 41% of the cells observed ($n=34$). After treatment with 10 nM CPA, mean calcium increase of 543 ± 97 nM was recorded in 100% of the cells observed ($n=33$).

6 These data indicate that adenosine A₁ receptors couple to intracellular calcium mobilization and extracellular calcium influx in type-1 and type-2 astrocytes in primary culture. In addition, the simultaneous activation of adenosine A₁ receptors on type-2 astrocytes results in an augmentation of the calcium response to histamine H₁ receptor stimulation.

Keywords: Rat astrocytes; intracellular calcium; adenosine A₁ receptors; histamine H₁ receptors

Introduction

In 1979, Van Calker *et al.* proposed the existence of adenosine A₁ and A₂ receptors which were respectively negatively and positively coupled to adenylyl cyclase via G_i- and G_s-proteins. The A₂ receptor subtype has since been further subdivided into A_{2a} and A_{2b} populations on the basis of the selective actions of the agonist, CGS 21680 (Jarvis *et al.*, 1989) and the antagonist, PD115,199 (Bruns *et al.*, 1987) at A₂ receptors in rat striatal membranes (Daly *et al.*, 1983). Support for this classification has been obtained from the molecular cloning and expression of proteins which appear to correspond to the rat (Mahan *et al.*, 1991), dog (Libert *et al.*, 1991), human (Libert *et al.*, 1992) and bovine (Olah *et al.*, 1992) A₁ receptors, the dog (Maenhaut *et al.*, 1990) and human (Furlong *et al.*, 1992) A_{2a} receptors and the rat (Rivkees & Reppert, 1992; Yakel *et al.*, 1993) and human (Pierce *et al.*, 1992) A_{2b} receptors. Recently novel proteins from rat (Zhou *et al.*, 1992), sheep (Linden *et al.*, 1993) and human (Salvatore *et al.*, 1993) brain have been cloned and expressed and are thought to represent a putative A₃ adenosine receptor binding site.

The presence of A₁ receptors with the ability to inhibit adenosine 3':5'-cyclic monophosphate (cyclic AMP) production via G_i-proteins has been demonstrated in various brain regions such as rat cerebral cortex, cerebellum, hippocampus and striatum (Ebersolt *et al.*, 1983). However, it is now clear that A₁ receptors can also couple to a variety of effector systems other than adenylyl cyclase. In peripheral tissues such as guinea-pig myometrium (Schiemann *et al.*, 1991), a rabbit cortical collecting tubule cell line (Arend *et al.*, 1989), the hamster vas deferens smooth muscle cell line, DDT₁MF-2 (White *et al.*, 1992) and in CHO-K1 cells expressing the human A₁ receptor transfect (Megson *et al.*, 1995), A₁ receptor stimulation causes the production of inositol phosphates. Furthermore, A₁ receptor-mediated increases in intracellular free calcium ion concentration have been reported in a renal epithelial cell line (Weinberg *et al.*, 1989), cultured mesangial cells (Olivera *et al.*, 1992), cortical collecting tubule cells (Arend *et al.*, 1988), tracheal epithelial cells (Galiotta *et al.*, 1992) and in both CHO-K1 cells expressing the transfected human A₁ receptor (Iredale *et al.*, 1994) and DDT₁MF-2 cells (Dickenson & Hill, 1993a), in which the effects were sensitive to pertussis toxin treatment. The precise mechanism for this response has not yet been determined; however, it may involve either a di-

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rect coupling of A₁ receptors to phospholipase C via a G_i-protein or the activation of phospholipase C by G_i-derived $\beta\gamma$ subunits (Dickenson & Hill, 1994).

Kriegler & Chiu (1993) have previously described transient elevations of $[Ca^{2+}]_i$ mediated by adenosine in glial cells of intact neonatal rat optic nerves injected with a fluorescent calcium indicator. However the receptor subtype involved in this response, was not characterized. In the present paper we have used subtype selective compounds combined with dynamic imaging techniques to investigate the coupling of adenosine A₁ receptors to the elevation of $[Ca^{2+}]_i$ in primary mixed astrocyte cultures. Furthermore, in type-2 astrocytes we have also studied the effects of A₁ receptor activation on the histamine H₁ receptor-mediated increases in intracellular calcium.

Methods

Cell culture

Primary cultures of mixed astrocytes were prepared by mechanical dissociation of tissue from 2 day old rat forebrains as previously reported (Peakman & Hill, 1994). Cells in 75 cm² flasks were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10% foetal calf serum (FCS) at 37°C in a water saturated atmosphere of 10% CO₂ in air. Penicillin 100 u ml⁻¹ and streptomycin 100 µg ml⁻¹ were also present during the first 24 h of culture. The medium was changed after 1 and 3 days *in vitro* and thereafter every 5 days.

Image analysis

After 13–17 days *in vitro*, cells were removed with trypsin-EDTA solution and reseeded, using a 1:2 or 1:3 split ratio, onto glass coverslips. Two days later, cells had become attached to the coverslips and had differentiated into the classic polygonal, type-1 and stellate, type-2 morphologies. The cells were washed 3 times with 2 ml physiological saline (PSS, composition, mM: NaCl 145, KCl 5.0, CaCl₂ 2.0, MgSO₄ 1.0 HEPES 10.0, glucose 10.0, adjusted to pH 7.4) and incubated at 37°C with 200 µl PSS containing 10% foetal calf serum and 5 µM fura-2 acetoxymethylester. After 1 h, excess dye was removed by a further 2 washes with 2 ml PSS. The coverslips were maintained in 2 ml PSS at 37°C in the dark and used within 2 h of loading.

Coverslips were mounted so that they formed the base of an open, cylindrical coverslip holder which was maintained at 37°C. The cells were bathed in 900 µl PSS or calcium-free PSS containing 0.1 mM EGTA. Where appropriate, the antagonist 8-cyclopentyl-1,3-dipropyladenosine (DPCPX, 0.1 µM) was included in this solution and incubated with the cells for 5–10 min before image capture. Dynamic video imaging was performed with MagiCal hardware and TARDIS software supplied by Applied Imaging International Ltd. (Hylton Park, Sunderland, Tyne and Wear) as previously described (Marsh & Hill, 1993; see also Neylon *et al.*, 1990 for detail). Two images, I (340 nm) and J (380 nm) were captured from an area of the coverslip which was devoid of cells in order to record the background fluorescence. An appropriate field of view containing type-1 and/or type-2 cells was then selected on the basis of cell morphology as observed through the eyepiece of the microscope. A sequence of 118 frames (each 256 × 256 pixels) was captured at alternating 340 nm (A frames) and 380 nm (B frames) wavelengths with a 1.5 s delay between each image pair. A 200 ms switch time between the two wave lengths was imposed by the hardware averaging of 8 consecutive images to produce each frame. Agonists were added in 100 µl PSS after approximately 14 A and B frames had been captured to provide basal measurements. When the effects of two agonists were investigated in the same capture sequence, the first agonist was added in 90 µl of PSS to a coverslip bathed in 810 µl PSS. The second agonist was then

added in 100 µl PSS as usual. At the end of sequence capture, frame I was subtracted from all A images and frame J from all B images. The corrected A and B frames were ratioed to produce R frames. Single cells in the R frames were defined by using a light pen and the average fluorescence intensity of all pixels in the defined region was calculated by TARDIS for each time point in the experiment. The average fluorescence intensities of pixels were correlated with calcium ion concentration using the Grynkiewicz *et al.* (1985) equation:

$$[Ca^{2+}] = K_d \beta [R - R_{min} / R_{max} - R]$$

where K_d = the dissociation constant (224 nM) for fura-2 and calcium at 37°C; R = the measured fluorescence ratio (intensity at 340 nm/intensity at 380 nm); R_{max} = the measured fluorescence ratio of Ca²⁺-saturated fura-2; R_{min} = the measured fluorescence ratio in the absence of Ca²⁺ ions; β = (the fluorescence intensity at 380 nm in the absence of Ca²⁺ ions)/(the fluorescence intensity at 380 nm of Ca²⁺-saturated fura-2)

Values for R_{max} and R_{min} were calculated by the addition of 100 µl PSS containing either 20 mM CaCl₂ and 40 µM ionomycin (for R_{max}) or 15 mM EGTA, 40 µM ionomycin and 0.1 M NaOH (for R_{min}). The two types of astrocytes in primary cultures were initially calibrated separately. The average values obtained for type-1 astrocytes were $R_{max} = 1.6 \pm 0.2$ ($n = 20$ cells, 10 separate coverslips), $R_{min} = 0.5 \pm 0.1$ ($n = 16$ cells, 8 separate coverslips) and $\beta = 4.4 \pm 1.7$. The average values obtained for type-2 astrocytes were $R_{max} = 1.6 \pm 0.1$ ($n = 41$ cells, 12 separate coverslips), $R_{min} = 0.5 \pm 0.05$ ($n = 34$ cells, 9 separate coverslips) and $\beta = 6.7 \pm 1.0$. Since the calibration values were so similar for each cell type, they were combined to produce values of $R_{max} = 1.6 \pm 0.1$ ($n = 61$ cells), $R_{min} = 0.5 \pm 0.05$ ($n = 50$ cells) and $\beta = 5.9 \pm 0.8$. Plots of calcium ion concentration against time were generated by TARDIS for each individual cell within the R frames.

Data analysis

Agonist concentration-response data were combined so that each data point represented the mean $\pm$ s.e.mean of the increase in $[Ca^{2+}]_i$ over basal levels from n cells on at least 3 separate coverslips derived from different preparations. These values were calculated using two different values for n so that the results were expressed either as a mean $\pm$ s.e.mean of all cells observed (regardless of whether they responded or not) or as a mean $\pm$ s.e.mean of only those cells which exhibited increases in $[Ca^{2+}]_i$. 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.

Statistical analysis was performed within single experiments by using Student's unpaired *t* test or one way analysis of variance (ANOVA) with *post-hoc* Newman-Keuls. Statistical analysis was performed between multiple experiments by using Student's paired *t* test or two way analysis of variance (ANOVA) with *post-hoc* Newman-Keuls.

Materials

Wistar rats were obtained from the Medical School Animal Unit, University of Nottingham. DMEM was obtained from Biological Industries (Cumbernauld, U.K.) and foetal calf serum was purchased from Advanced Protein Products (Brierley Hill, U.K.). Fura-2 acetoxymethylester was purchased from Calbiochem Novabiochem Corp., Beeston, Nottingham. N⁶-cyclopentyladenosine and histamine were supplied by Sigma Chemical Co. (Poole U.K.). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) was supplied by Research Biochemicals Inc. Semat (St. Albans, U.K.).

Results

Imaging experiments were performed using coverslips containing populations of both type-1 and type-2 astrocytes as described in the Methods. The calcium responses of each cell type were then analysed separately by use of a light pen to outline single cells within each field of view. In this section, the results obtained for each astrocyte subtype have been considered individually.

Type-2 astrocytes

Type-2 astrocytes in cultures containing a mixture of type-1 and type-2 populations had a mean basal $[Ca^{2+}]_i$ of 192 ± 5 nM ($n=697$ cells). In the presence of extracellular calcium, the application of high concentrations of histamine caused a large elevation in this level. Figure 1a and 1b shows examples of traces obtained from single cells stimulated with either 1 mM or 10 μ M histamine respectively. The peak $[Ca^{2+}]_i$ attained in each type-2 cell was recorded and the size of this response was found to be concentration-dependent (Figure 2). In Figure 2a the effects of increasing concentrations of histamine are expressed as the mean increases in $[Ca^{2+}]_i$ over basal levels in all type-2 astrocytes which were visible in the captured frames. However, a number of cells did not respond to histamine with an increase in $[Ca^{2+}]_i$ and therefore, Figure 2b shows the mean values calculated from responding cells only. An apparently maximal response was achieved with histamine at concentrations between 1 μ M and 1 mM (1 mM histamine, mean $[Ca^{2+}]_i$ increase in responding cells = $1,468 \pm 94$ nM over basal levels,

$n=44$). A much lower response, however, was obtained with 0.1 μ M histamine (mean $[Ca^{2+}]_i$ increase in responding cells = 337 ± 34 nM over basal levels, $n=26$). The percentage of

Figure 1 Effect of histamine (Hist) on the intracellular calcium ion concentration in single type-2 astrocytes shown in real time. (a) Traces represent the changes in $[Ca^{2+}]_i$ in 2 single cells in the same field of view, in response to treatment with 1 mM histamine added at the arrow. (b) Traces represent the changes in $[Ca^{2+}]_i$ in 3 single cells in the same field of view, in response to treatment with 10 μ M histamine added at the arrow.

Figure 2 Mean effects of histamine on the elevation of intracellular calcium ion concentration in single type-2 astrocytes. Numbers inside the columns indicate the number of cells analysed, from at least 3 separate animal preparations, in order to generate the value at that agonist concentration. (a) Values represent the means $\pm$ s.e. means of the maximal increases in $[Ca^{2+}]_i$ over basal in all visible type-2 astrocytes. (b) Values represent the means $\pm$ s.e. means of the maximal increases in $[Ca^{2+}]_i$ over basal in responding type-2 astrocytes only.

Table 1 Frequency of the intracellular calcium responses to histamine and N⁶-cyclopentyladenosine in single type-2 astrocytes

Agonist (M)	Frequency of response (%)	
	Histamine	N ⁶ -cyclopentyladenosine
10^{-3}	90 (44/49)	NR
10^{-4}	88 (28/32)	NR
10^{-5}	88 (28/32)	30 (16/53)
10^{-6}	62 (28/45)	31 (32/104)
10^{-7}	39 (26/67)	11 (4/36)
10^{-8}	NR	17 (14/82)
10^{-9}	NR	22 (4/18)

The frequency of response was obtained from cells on at least 3 separate coverslips derived from different primary mixed astrocyte preparations and was calculated by expressing the number of responding type-2 astrocytes as a percentage of the total number of type-2 astrocytes observed. The numbers in parentheses indicate the number of responding type-2 cells/the total number of type-2 cells observed. NR, not recorded.

responding cells at each agonist concentration is shown in Table 1. Calcium elevations were recorded in approximately 90% of cells after stimulation with 10 μ M histamine or above, however, only 39% of the 67 cells treated with 0.1 μ M histamine responded with increases in $[Ca^{2+}]_i$. Typical traces from 3 type-2 cells which did not respond to stimulation with 0.1 μ M histamine are shown in Figure 8a. The traces in Figure 1 indicate that the temporal pattern of the response to histamine was not the same in all cells. Figure 3 shows example traces from individual cells which illustrate the types of response observed. The most frequent response, recorded in 68% of the 154 cells responding to histamine, was a dramatic, transient peak in $[Ca^{2+}]_i$ which declined to basal (Figure 3a) or to some

Figure 3 Typical examples of the alterations in intracellular calcium ion concentration observed in type-2 astrocytes after treatment with 0.1 mM histamine (Hist). The most frequent response was a dramatic elevation in $[Ca^{2+}]_i$ which was either transient, declining quickly to basal values (a) or biphasic, declining to some persistently elevated level (b). On a number of occasions the elevation of $[Ca^{2+}]_i$ was sustained for the remainder of the experiment (c).

Figure 4 Effect of N⁶-cyclopentyladenosine on the intracellular calcium ion concentration in single type-2 astrocytes shown in real time. Traces represent the changes in $[Ca^{2+}]_i$ in 3 single cells in the same field of view, in response to the addition of 1 μ M N⁶-cyclopentyladenosine (CPA) at the first arrow followed by the further addition of 10 μ M histamine (Hist) at the second arrow. Two cells responded to both CPA and histamine. One cell did not exhibit an increase in $[Ca^{2+}]_i$ in response to CPA but did subsequently respond to histamine.

Figure 5 Mean effects of N⁶-cyclopentyladenosine on the intracellular calcium ion concentration in single type-2 astrocytes. Numbers inside the columns indicate that the number of cells analysed, from at least 3 separate animal preparations, in order to generate the value at the agonist concentration. (a) Values represent the means $\pm$ s.e. means of the maximal increases in $[Ca^{2+}]_i$ over basal in all visible type-2 astrocytes. (b) Values represent the means $\pm$ s.e. means of the maximal increases in $[Ca^{2+}]_i$ over basal in responding type-2 astrocytes only.

Figure 6 Typical examples of the alterations in intracellular calcium ion concentration observed in type-2 astrocytes after treatment with various concentrations of N⁶-cyclopentyladenosine. Responses were generally spiky and predominantly transient (a) or oscillatory (b). Sustained (c) and biphasic (d) responses were also observed.

lower but persistently elevated level (Figure 3b). A sustained increase in $[Ca^{2+}]_i$ was observed in 21% of responding cells (Figure 3c). The shape of the calcium response observed did not appear to correlate with agonist concentration.

Figure 7 Effect of 8-cyclopentyl-1,3-dipropylxanthine or the absence of extracellular calcium on the N⁶-cyclopentyladenosine-mediated increases in intracellular calcium ion concentration in single type-2 astrocytes shown in real time. Traces represent the changes in $[Ca^{2+}]_i$ in single type-2 astrocytes on separate coverslips derived from the same animal preparation and analysed on the same day, in response to the addition of 1 μM N⁶-cyclopentyladenosine (CPA) at the first arrow followed by the further addition of 1 mM histamine (Hist) at the second arrow. (a) Traces show that in the absence of extracellular calcium (EGTA 0.1 mM) elevations in $[Ca^{2+}]_i$ were still generated by both CPA and histamine. (b) The control trace for a single type-2 astrocyte in the presence of extracellular calcium and the absence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) reveals responses to both agonists. (c) Traces show that in the presence of extracellular calcium and after 10 min pretreatment with 0.1 μM DPCPX, three cells did not respond to 1 μM CPA with an increase in $[Ca^{2+}]_i$ but subsequently responded to 1 mM histamine. In addition, one cell did not respond to either CPA or histamine.

Table 2 Characteristics of the intracellular calcium responses to a combination of histamine (0.1 μ M) and N⁶-cyclopentyladenosine (10 nM) in type-2 astrocytes

Agonist	Mean $[Ca^{2+}]_i$ increase (nM)		Frequency of response (%)
	All cells	Responding cells	
Histamine (0.1 μ M)	108 $\pm$ 25	268 $\pm$ 34	41 (14/34)
CPA (10 nM)	543 $\pm$ 97	543 $\pm$ 97	100 (33/33)
+ histamine			

The mean increase in intracellular calcium ion concentration was calculated both for all type-2 astrocytes observed in each field (*All cells*) of view and for just those cells which responded to histamine or a combination of N⁶-cyclopentyladenosine (CPA) and histamine (*Responding cells*). The frequency of response was obtained from cells on 5 separate coverslips derived from 3 different animal preparations and was calculated by expressing the number of responding type-2 astrocytes as a percentage of the total number of type-2 astrocytes observed. The numbers in parentheses indicate the number of responding type-2 cells/total number of type-2 astrocytes observed. Where appropriate CPA was applied prior to addition of histamine (see Figure 8). No change in $[Ca^{2+}]_i$ was observed in response to the application of CPA in 32 of the 33 cells observed. In one cell, CPA produced a calcium elevation of 611 nM.

Figure 8 Effect of N⁶-cyclopentyladenosine on the intracellular calcium ion concentration in histamine-treated single type-2 astrocytes shown in real time. (a) Traces represent the effect of 0.1 μ M histamine (Hist, added at the arrow) on 3 single type-2 astrocytes. No responses were visible. (b) A second coverslip derived from the same animal preparation and analysed on the same day as the coverslip in (a) was used to investigate the effects of N⁶-cyclopentyladenosine (CPA) pretreatment. Traces represent the changes in $[Ca^{2+}]_i$ in 3 single type-2 astrocytes in response to the addition of 10 nM CPA at the first arrow followed by the further addition of 0.1 μ M histamine at the second arrow. The addition of 10 nM CPA had little or no direct effect on $[Ca^{2+}]_i$, however, a significant augmentation of the effects of 0.1 μ M histamine was seen in all cells.

In further studies, in the presence of extracellular calcium, the adenosine A₁ receptor-selective agonist, N⁶-cyclopentyladenosine (CPA) also caused an elevation of $[Ca^{2+}]_i$ in some type-2 astrocytes. In 16 cells (30%) out of a total of 53 which were observed, 10 μ M CPA increased $[Ca^{2+}]_i$ by a mean value of $1,110 \pm 181$ nM over basal levels. Examples of the profiles obtained from 3 single type-2 cells after stimulation with 1 μ M

CPA are shown in Figure 4. Rapid spikes in $[Ca^{2+}]_i$ were generated by two cells whereas a third cell did not appear to exhibit a calcium response. Since the frequency of response was much lower than had previously been observed for histamine, a relatively high concentration of histamine was added to the cells towards the end of each experiment in order to provide some indication of their viability. In Figure 5a the effects of a range of concentrations of CPA are expressed as the mean increases in $[Ca^{2+}]_i$ in all type-2 astrocytes which were visible in the captured frames. These results are expressed in Figure 5b as the mean increases in $[Ca^{2+}]_i$ in only those type-2 cells which responded to CPA. The elevations in $[Ca^{2+}]_i$ were apparently concentration-dependent and the frequency of responding cells at each agonist concentration is shown in Table 1. A maximal responding frequency of approximately 30% was recorded after stimulation with 1 μ M ($n = 104$ cells in total) or 10 μ M ($n = 53$ cells in total) CPA; however, there did not appear to be a concentration-dependent increase in the frequency of the response up to this level. The profiles in Figure 6 provide typical examples of the responses to CPA observed in individual cells. Approximately 65% of the 70 CPA-mediated calcium responses observed were rapid and spiky in appearance, the majority of these being transient in nature (Figure 6a). In approximately 21% of the responding cells, oscillatory responses with a mean frequency of 3.3 ± 0.4 min⁻¹ ($n = 15$ cells) were recorded (Figure 6b). Sustained elevations of $[Ca^{2+}]_i$ were observed in approximately 16% of responding cells (Figure 6c) and the remaining responses tended to be biphasic with a rapid elevation of $[Ca^{2+}]_i$ followed by a slower decline to basal levels (Figure 6d). No apparent relationship was evident between the agonist concentration and the shape of the resulting response.

The CPA-mediated response in type-2 astrocytes was further investigated either in the presence of A₁ receptor-selective antagonist, DPCPX, or in the absence of extracellular calcium. A protocol was adopted using 3 consecutive coverslips of cells derived from the same animal preparation so that, as far as was possible, direct comparisons could be made between coverslips. The first coverslip (in the absence of DPCPX) was stimulated with 1 μ M CPA in calcium-free PSS (containing 0.1 mM EGTA). The second coverslip was stimulated with 1 μ M CPA in the absence of DPCPX and in calcium-containing PSS. The third coverslip (in calcium-containing PSS) was stimulated with 1 μ M CPA in the presence of 0.1 μ M DPCPX. This design enabled the second coverslip, to be considered as a control for both the first and the third coverslips. In 5 separate control coverslips derived from 3 different animal preparations, 1 μ M CPA, produced a mean increase in $[Ca^{2+}]_i$ of 975 ± 189 nM over basal levels in 31% (10) of 32 cells observed. Figure 7b shows responses to 1 μ M CPA and 1 mM histamine obtained from a single type-2 cell on a typical control coverslip.

In the presence of extracellular calcium, the CPA-mediated elevation of $[Ca^{2+}]_i$ in type-2 astrocytes was antagonized by the A₁ receptor-selective antagonist, DPCPX. In 28 type-2 cells on 5 coverslips pretreated with 0.1 μ M DPCPX for 5–10 min, no

Table 3 Characteristics of the intracellular calcium responses to histamine and N⁶-cyclopentyladenosine (CPA) in single type-1 astrocytes

Agonist	(M)	Mean [Ca ²⁺] _i increase (nM)		Frequency of response (%)
		All cells	Responding cells	
Histamine	10 ⁻³	268 ± 99	805 ± 197	33 (8/24)
	10 ⁻⁴	337 ± 127	1123 ± 300	30 (9/30)
CPA	10 ⁻⁵	141 ± 50	605 ± 89	23 (7/30)
	10 ⁻⁶	0	0	0 (0/33)

The mean increase in intracellular calcium ion concentration was calculated both for all type-1 astrocytes observed in each field (*All cells*) of view and for just those cells which responded to the agonist (*Responding cells*). The frequency of response was obtained from cells on at least 3 separate coverslips derived from different primary mixed astrocyte preparations and was calculated by expressing the number of responding type-1 astrocytes as a percentage of the total number of type-1 astrocytes observed. The numbers in parentheses indicate the number of responding type-1 cells/the total number of type-1 cells observed.

increases in [Ca²⁺]_i were recorded in response to 1 μM CPA. The traces in Figure 7c were obtained from 4 cells on a separate coverslip which were derived from the same animal preparation as the cell in Figure 7b and analysed immediately after it. The cells were pretreated with DPCPX for 10 min and then stimulated with 1 μM CPA followed by 1 mM histamine. Three of the cells did not exhibit calcium responses to CPA but did subsequently respond to histamine. One cell did not respond to either CPA or histamine (Figure 7b). The mean increase in [Ca²⁺]_i obtained in response to histamine (1 mM) following DPCPX (0.1 μM) treatment in responding cells (1907 ± 119 nM; *n* = 11), and the frequency of responding cells (85%; 11/13), were similar to the values obtained in the absence of DPCPX (Table 1, Figure 2b).

In the absence of extracellular calcium ions there was a significant drop (*P* < 0.001, Student's unpaired *t* test) in the mean basal level of [Ca²⁺]_i in type-2 astrocytes from 192 ± 5 nM (*n* = 607 cells), calculated in the presence of extracellular calcium, to 141 ± 7 nM (*n* = 103 cells). Under these conditions, however, CPA was still able to elicit increases in [Ca²⁺]_i in type-2 astrocytes. On 5 separate coverslips stimulated with 1 μM CPA in the absence of extracellular calcium and in the presence of 0.1 mM EGTA, a mean elevation in [Ca²⁺]_i of 504 ± 67 nM over basal levels was observed in 41% (17) of 41 cells analysed. Figure 7a shows profiles obtained from two individual cells which were derived from the same animal preparation as the cell in Figure 7b. The cells were bathed in calcium-free medium and then stimulated with 1 μM CPA followed by 1 mM histamine. One cell produced large responses to both CPA and histamine. The second cell gave a large response to CPA but subsequently only a small response to histamine.

The effect of CPA on the response to a low histamine concentration was studied. Coverslips of cells derived from the same animal preparation were alternatively stimulated with either 0.1 μM histamine alone or with 10 nM CPA followed by 0.1 μM histamine. In 5 separate control coverslips from 3 different animal preparations, treatment with 0.1 μM histamine alone caused a mean increase in [Ca²⁺]_i of 268 ± 34 nM over basal levels in 41% (14) of 34 type-2 cells analysed (Table 2). In 5 corresponding coverslips pretreated with 10 nM CPA, all of the 33 cells observed, responded to 0.1 μM histamine with a mean increase in [Ca²⁺]_i of 543 ± 97 nM over basal levels (Table 2). In these experiments, however, a calcium elevation of 611 nM over basal levels was only recorded in a single cell in response to treatment with 10 nM CPA. The other cells were insensitive to the first application of CPA. This was notably lower than the 17% responding frequency (see Table 1) previously observed at this concentration of CPA. Figure 8 shows examples of responses observed in corresponding coverslips from the same animal preparation. In Figure 8a traces from 3 cells show no elevations of [Ca²⁺]_i in response to 0.1 μM histamine alone. In Figure 8b the addition of 10 nM CPA to a separate coverslip appeared to cause a small increase in [Ca²⁺]_i

in one cell but to have no effect on 2 other cells. Subsequent treatment with 0.1 μM histamine resulted in a much augmented effect and elevations of [Ca²⁺]_i were recorded in all 3 cells.

Type-1 astrocytes

In cultures containing mixed type-1 and type-2 astrocyte populations a mean basal [Ca²⁺]_i of 207 ± 8 nM (*n* = 254 cells) was calculated for type-1 cells. At high concentrations, histamine caused elevations of [Ca²⁺]_i in approximately 30% of the type-1 astrocytes observed. The data obtained are summarized in Table 3. The application of 1 mM histamine elicited a mean [Ca²⁺]_i elevation of 805 ± 197 nM over basal levels in 8 of 24 type-1 cells observed on 6 separate coverslips derived from 5 different animal preparations. A mean [Ca²⁺]_i increase of 605 ± 89 nM over basal levels was also recorded in 23% of type-1 astrocytes on 7 separate coverslips derived from 5 different animal preparations which were stimulated with 10 μM CPA (*n* = 30). When 1 μM CPA was used, no calcium responses were observed. Table 3 summarises the data which were obtained.

Discussion

Previous investigations in our laboratory have shown that in the hamster-derived DDT₁MF-2 cell line and in CHO-K1 cells expressing the human A₁ receptor transfect, A₁ receptor activation causes the stimulation of phospholipase C resulting in an elevation of inositol phosphate accumulation and a subsequent increase in [Ca²⁺]_i (White *et al.*, 1992; 1993; Dickenson & Hill, 1993a; Iredale *et al.*, 1994). Furthermore, in the smooth muscle cell line, A₁ receptor-mediated potentiations of the calcium responses elicited by low concentrations of histamine have also been reported (Dickenson & Hill, 1993b). In slices of guinea-pig cerebral cortex, A₁-like receptor activation appears to potentiate histamine H₁ receptor-mediated phosphoinositide turnover (Hollingsworth *et al.*, 1986); however, the cell type involved in this interaction remains to be defined. In the present studies we have therefore investigated the effect of A₁ receptor activation on [Ca²⁺]_i in rat-derived type-1 and type-2 astrocytes in culture.

Previous studies investigating calcium responses in type-1 astrocytes have reported that in 3–9% of cells, spontaneous oscillations in basal [Ca²⁺]_i with a frequency of 1.2–1.8 min⁻¹ were observed (Nilsson *et al.*, 1991a,b; Helen *et al.*, 1992). In these experiments the authors noted that agonist addition often caused an increase in the frequency and magnitude of these fluctuations. One out of 23 type-2 astrocytes examined by Dave *et al.* (1991) exhibited similar spontaneous oscillations although the frequency of these was not reported. In the present studies, spontaneous oscillations were observed in a total of 5 type-2 astrocytes (*n* = 697) but in no type-1 cells (*n* = 254). Unfortunately, the frequency of these fluctuations

could not be measured as only one spike in $[Ca^{2+}]_i$ was observed prior to agonist addition (at approximately 20 s) in each case. Four of the oscillating cells were treated with CPA (1 μ M or 10 μ M) and of these, three responded with an increase in the frequency of oscillations and one produced a sustained $[Ca^{2+}]_i$ increase.

Histamine at concentrations of 0.1 μ M and above caused elevations of $[Ca^{2+}]_i$ in some type-2 astrocytes. Previously, these effects have been shown to be the result of intracellular calcium mobilisation and extracellular calcium influx mediated via the stimulation of H₁ receptors coupled in a pertussis toxin-insensitive manner to phospholipase C and inositol phosphate production (Fukui *et al.*, 1991; Inagaki *et al.*, 1991b). However, even after the application of 1 mM histamine, a proportion of type-2 astrocytes did not appear to respond in this manner. The frequency of responding cells at each agonist concentration was in close agreement with the values of 62% at 1 μ M and 73% at 10 μ M reported by Inagaki *et al.* (1991b). A constant maximum responding frequency of approximately 90% was recorded over the concentration range of 10 μ M to 1 mM which suggests that about 10% of type-2 astrocytes in the neonatal rat forebrain do not express functional histamine H₁ receptors coupled to phospholipase C. These observations support earlier works which have described a heterogeneous expression of neurotransmitter receptors in populations of the two astrocyte subtypes (Lerea & McCarthy, 1989; Dave *et al.*, 1991; Inagaki *et al.*, 1991a,b; Jensen & Chiu, 1991; McCarthy & Salm, 1991; Nilsson *et al.*, 1991a,b). When the sizes of the elevations in $[Ca^{2+}]_i$ were combined to produce mean responses for all the cells observed at a specific histamine concentration, they were found to increase in a concentration-dependent manner. The EC₅₀ value for histamine appeared to be between 0.1–1 μ M which was somewhat lower than the value of approximately 10 μ M which has previously been observed for this effect (Fukui *et al.*, 1991; Inagaki *et al.*, 1991b). The contribution of non-responding cells to the relationship was indicated by combining the data from responding cells only. The increasing recruitment of responsive cells with increasing histamine concentration may indicate that different cells express receptors which possess variable agonist sensitivities. The temporal patterns of histamine-mediated calcium responses were similar in nature to those previously described by Fukui *et al.* (1991) who classified them into transient, oscillatory, sustained and biphasic (transient peak declining to a persistently elevated level) patterns of response. This group suggested that oscillatory and transient responses were predominantly mediated by intracellular calcium release whereas extracellular calcium influx had greater importance in biphasic and sustained responses. At all concentrations of histamine investigated in the studies described here, the predominant responses were sustained or biphasic and no purely oscillatory responses were observed.

Histamine-stimulated $[Ca^{2+}]_i$ elevations were only apparent in 30% of type-1 astrocytes treated with 0.1 mM agonist indicating that histamine H₁ receptors functionally coupled to phospholipase C are predominantly expressed by type-2 astrocytes. These data concur with earlier studies which have recorded calcium elevations in only 25% (McCarthy & Salm, 1991) and 17% (Fukui *et al.*, 1991) of cortical type-1 astrocytes stimulated with 0.1 mM histamine and have shown no effect of histamine on inositol phospholipid hydrolysis (Fukui *et al.*, 1991; Kondou *et al.*, 1991).

In type-2 astrocytes concentration-dependent elevations of $[Ca^{2+}]_i$ were recorded after treatment with CPA at concentrations of 1 nM or above. The percentage of cells responding with calcium increases was lower than previously seen with histamine, reaching a maximum of approximately 30% at 1 μ M CPA, and did not appear to be dependent on agonist concentration. The temporal changes in $[Ca^{2+}]_i$ produced by CPA in type-2 astrocytes tended to be more spiky than those produced by histamine and transient or oscillatory profiles were notably more frequent. These effects appeared to be mediated via the stimulation of adenosine A₁ receptors since

they were completely abolished by pretreatment of the cells with 0.1 μ M DPCPX, an A₁ receptor-selective antagonist. In the absence of extracellular calcium the response to 1 μ M CPA was still observed. However, under these conditions, the mean increase in $[Ca^{2+}]_i$ calculated from 17 responding cells was significantly reduced to 51.7% ($P < 0.01$ Student's unpaired *t* test) of the mean increase calculated from 10 responding cells on matched coverslips in calcium containing buffer. Similar effects on the histamine-induced elevation of $[Ca^{2+}]_i$ in DDT₁MF-2 cells have been reported (Dickenson & Hill, 1991; 1992) although, in contrast, the CPA-mediated increases of $[Ca^{2+}]_i$ in this cell line were unaffected by removal of extracellular calcium (White *et al.*, 1992). The data imply that the CPA response in type-2 astrocytes has two components: (1) the release of calcium from intracellular stores and (2) influx of extracellular calcium through calcium channels in the plasma membrane. Numerous reports are now appearing which describe A₁ receptor coupling to phosphoinositide turnover (Arend *et al.*, 1988; Schiemann *et al.*, 1991) or the elevation of $[Ca^{2+}]_i$ (Arend *et al.*, 1988; Weinberg *et al.*, 1989; Galletta *et al.*, 1992; Olivera *et al.*, 1992) in peripheral cell lines and tissues. Adenosine has also been observed to cause transient calcium elevations in glial cells of rat intact neonatal optic nerves (Kriegler & Chiu, 1993). These experiments, provide the first description of A₁ receptor-mediated calcium responses in primary cultures of astrocytes derived from the CNS.

CPA induced elevations of $[Ca^{2+}]_i$ in only a small proportion (23%) of type-1 astrocytes even when used at a concentration of 10 μ M. These data suggest that in rat forebrain cultures, adenosine A₁ receptors are predominantly located on type-2 and not type-1 astrocytes. In previous experiments we have shown a lack of A₁ receptor-mediated inhibition of elevated [³H]-cyclic AMP levels in forebrain-derived type-1 astrocytes (unpublished observations) providing further support for this proposal. A recent study by Ogata *et al.* (1994) which reported the presence of adenosine A₁ receptors on type-1 astrocytes derived from embryonic rat hippocampi may therefore indicate that glial A₁ receptor expression varies with brain region.

The calcium response induced in type-2 astrocytes by low concentrations of histamine (0.1 μ M) was significantly potentiated by prior treatment with a low (10 nM) concentration of CPA. Two separate effects were apparent. The frequency of responding cells on matched coverslips increased from 41% ($n = 34$) responding to 0.1 μ M histamine alone, to 100% ($n = 33$) after pretreatment with 10 nM CPA. In addition, the mean increase in $[Ca^{2+}]_i$ calculated for the 33 cells pretreated with CPA, was double that calculated for the 14 cells responding to 0.1 μ M histamine alone. A similar augmentation of calcium responses to submaximal concentrations of histamine has been described in DDT₁MF-2 cells after prior treatment with maximal or submaximal concentrations of CPA (Dickenson & Hill, 1993b). Similar effects were reported in slices of guinea-pig cerebral cortex where adenosine analogues which had no effect on phosphoinositide turnover (Brown *et al.*, 1984) potentiated the histamine H₁ receptor-mediated response (Hollingsworth *et al.*, 1986). More recently, Alexander *et al.* (1994) have demonstrated that activation of metabotropic glutamate receptors can likewise augment histamine-induced inositol phosphate accumulation in the guinea-pig brain.

The effects of CPA on the calcium response to histamine were not examined in type-1 astrocytes because of the low responding frequencies observed after stimulation with each agonist alone. It is therefore interesting that in hippocampal astrocytes which had the morphology of type-1 cells, the elevation of $[Ca^{2+}]_i$ caused by metabotropic glutamate receptor activation was recently augmented by the stimulation of receptors which were apparently of the A₁ subtype (Ogata *et al.*, 1994).

The mechanism(s) for the potentiations which have been reported has not yet been fully elucidated but it appears to require the presence of receptor systems coupled to different G-proteins. Hence in DDT₁MF-2 cells the pertussis toxin-sensi-

tive A₁ receptor-mediated effect has additionally been shown to augment responses to ATP (Gerwins & Fredholm, 1992a) and bradykinin (Gerwins & Fredholm, 1992b) which, like histamine (Dickenson & Hill, 1993a,b) mediate their effects through receptors coupled to phospholipase C via pertussis toxin-insensitive G-proteins. In addition, in mouse striatal astrocytes, somatostatin and A₁ receptors which couple to pertussis toxin-sensitive G-proteins, both interact synergistically with the pertussis toxin-insensitive, α_1 -adrenoceptor-mediated increase in inositol phosphate accumulation (El-Etr *et al.*, 1992a,b; Marin *et al.*, 1993). Since the H₁ receptor-mediated response in type-2 astrocytes has previously been shown to be pertussis toxin-insensitive (Fukui *et al.*, 1991), it appears likely that the CPA response in these cells is effected via a pertussis toxin-sensitive G-protein but this remains to be established. In DDT₁MF-2 cells, these interactions have been suggested to involve extracellular and intracellular calcium, protein kinases A and C, and the products of arachidonic acid metabolism (Gerwins & Fredholm, 1992a,b). The cross-talk between A₁ receptors and α_1 -adrenoceptors in mouse astrocytes appears to be mediated via the inhibition of glutamate reuptake into astrocytes by arachidonic acid and a similar mechanism may mediate the effects of adenosine on glutamate receptor-mediated calcium responses in hippocampal astro-

cytes (Ogata *et al.*, 1994), although the latter effect, unlike the former is independent of extracellular calcium. The activity of G_i-derived $\beta\gamma$ subunits which can stimulate some isoforms (mainly β_2 and β_3) of phospholipase C (see Clapham & Neer, 1993) has also been proposed but has yet to be fully investigated (Dickenson & Hill, 1994b).

In conclusion, the results of this study support earlier indications that adenosine A₁ receptors in glial cultures are primarily located on type-2 astrocytes and demonstrate that in these cells, A₁ receptor activation can cause intracellular calcium mobilisation and extracellular calcium influx. In addition, the data provided evidence that adenosine A₁ and histamine H₁ receptors on astrocytes interact synergistically to raise levels of [Ca²⁺]_i and therefore indicate that glial cells may be responsible, at least in part, for interactions which have previously been reported in brain slices.

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