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α- AND β-ADRENOCEPTOR REGULATION OF CYCLIC AMP ACCUMULATION IN CULTURED RAT ASTROCYTES

A COMPARISON OF PRIMARY PROTOPLASMIC AND MIXED FIBROUS/PROTOPLASMIC ASTROGLIAL CULTURES

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Abstract—The effect of noradrenaline and isoprenaline on cyclic AMP accumulation has been investigated in primary rat astrocytes which contain either (a) protoplasmic astrocytes alone or (b) both fibrous and protoplasmic astrocytes. Isoprenaline and noradrenaline stimulated cyclic AMP formation in both astrocyte culture preparations. Combinations of noradrenaline (1 μM) and isoprenaline (1 μM) produced a cyclic AMP response which was 58% and 26% of that produced by isoprenaline alone in protoplasmic and mixed fibrous/protoplasmic cultures, respectively. In both preparations this inhibitory effect of noradrenaline was antagonized by the α_2-adrenoceptor antagonist yohimbine (1 μM). A striking feature of the concentration–response curve for isoprenaline ($EC_{50} = 0.8$ μM) in mixed fibrous/protoplasmic cultures was that the cyclic AMP response decreased sharply at concentrations above 1 μM. This phenomenon was not seen in cultures containing protoplasmic astroglia alone. The fall in the isoprenaline concentration–response curve was not observed in the presence of the α-adrenoceptor antagonist phentolamine (1 μM), the dihydropyridine calcium antagonist isradipine (10 μM), the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.1 mM) or in nominally calcium-free medium. The effect of phentolamine was mimicked by the α_1-adrenoceptor antagonist prazosin (1 μM) but not by the α_2-antagonist yohimbine (1 μM). In conclusion, the data from this study suggest that two different populations of astrocytes in *in vitro* culture are able to raise intracellular cyclic AMP levels via β-adrenoceptor activation and that there are differences in the extent of α-adrenoceptor (both α_1- and α_2-) mediated inhibition of cyclic AMP accumulation between the two primary astroglial cell preparations.

A number of previous studies, including those from this laboratory, have been concerned with receptors that modulate the functional responses of other receptor systems [1]. An example is the interaction between adrenoceptors in rat brain in which cyclic AMP accumulation due to β-adrenoceptor stimulation is both potentiated by one population of α-adrenoceptors [2–4] and inhibited by a different population of α-adrenoceptors [5–6].

These studies, which used brain slices as an experimental model, have proved somewhat difficult to interpret, however, because of the heterogeneous cellular nature of the slices and the uncertain distribution of α- and β-adrenoceptors. More recently, primary cultures of brain cells and tumour cells of CNS origin have been used to investigate various neurotransmitter responses at the receptor level [7–9].

There is now considerable evidence that astrocytes derived from neonatal rodent brain possess both α- and β-adrenoceptors [10–13]. The β-adrenoceptors are coupled positively to adenylate cyclase leading to a rise in intracellular cyclic AMP levels upon receptor stimulation [7, 14]. Astroglia prepared from newborn rat cerebral cortex have been shown by ligand binding studies to possess mainly the β_1-adrenoceptor subtype [15] while there is evidence from studies of both ligand binding and cyclic AMP accumulation that astrocytes derived from mouse brain possess both β_1- and β_2-adrenoceptors [16, 17].

Electrophysiological and ligand binding studies indicate that the α-adrenoceptors present on astroglial cells are of both the α_1- and α_2-subtype [11, 12]. The α_1-adrenoceptors are linked to inositol phospholipid hydrolysis [18–20] while stimulation of α_2-adrenoceptors reduces the β-adrenoceptor-induced increase in cyclic AMP accumulation [7–9]. The presence of both α- and β-adrenoceptors on astrocytes in the brain may be of significance in the regulation of the response to noradrenaline released from neurons, since neurons prepared from foetal tissue do not appear to possess β-adrenoceptors [14].

Two morphologically distinct cell types, which appear to represent different stages of astroglial differentiation, can be prepared from the neonatal rat brain [21, 22]. The predominant cell type consists of flat polygonally shaped (protoplasmic or type 1) astroglial cells while the second cell type has a central body from which short processes emanate (fibrous or type 2) and grows on top of a bed layer of protoplasmic cells [22]. Both cell types have been identified in *in vitro* cell culture primarily on the basis of the content of glial fibrillary acidic protein

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which is astrocyte specific in brain [23]. Therefore it seems that astrocytes may also be a heterogenous population of cells which may in turn possess differences in the distribution and expression of certain neurotransmitter receptors. For example, ligand binding studies combined with immunofluorescent techniques have indicated that type 2 or fibrous astroglia do not possess β-adrenoceptors on their cell surface [14]. This finding has particular relevance to the recent observation that certain β-adrenoceptor agonists can promote differentiation of astrocytes in culture leading to a change in morphology from a protoplasmic to the fibrous state [24].

Previous studies of the regulation on cyclic AMP accumulation in primary rat astrocytes by α- and β-adrenoceptors have been restricted to cultures which are likely to contain both fibrous and protoplasmic astroglia [7–9, 14]. We have therefore undertaken a comparison of the effects of α- and β-adrenoceptor stimulation on cyclic AMP accumulation in cultures of primary rat astrocytes which contain either (a) protoplasmic astrocytes alone or (b) both fibrous and protoplasmic astrocytes.

MATERIALS AND METHODS

Cell culture. Glial cell cultures were prepared from 2 (mixed protoplasmic/fibrous cultures) or 8 day old (protoplasmic cultures) rat brain frontal lobes by a modification of the methods of McCarthy and de Vellis [21] and Ebersolt *et al.* [16, 17]. Briefly, animals were killed and the brains removed under sterile conditions. After removal of the meninges, the tissue was dissociated mechanically through nylon meshes (pore sizes 250 and 150 μm) to obtain a single cell suspension. Cells were plated at a density of 5×10^5 cells/cm^3 into 70 cm tissue culture flasks. Cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM glutamine and 10% foetal calf serum (FCS) at 37° in a water saturated atmosphere of 10% CO_2 in air. Cultures were left undisturbed for 24 hr, thereafter the medium was changed every 3–4 days. The cells were allowed to grow for up to 10 days *in vitro* by which time they had obtained confluence. The cultures were then shaken overnight on a mechanical shaker to remove the oligodendroglia. Following shaking, the cells were trypsinized and plated (plating density 5×10^4 cells per well) into 24-well cluster dishes at least 24–48 hr before cyclic AMP accumulation experiments were performed.

Cell identification. To check the purity of the prepared cultures, cells were plated (plating density 5×10^4 cells) onto 13 mm glass coverslips. At confluence, the cells were fixed in 3.8% formaldehyde and stained with antibodies to the intermediate filament proteins, glial fibrillary acidic protein (GFAP) and vimentin, using indirect immunofluorescence. The relative proportions of fibrous and protoplasmic cells in mixed cultures was based on a cell count of the number of cells exhibiting process-bearing morphology and GFAP immunoreactivity per field of 100 cells. In these mixed cultures 64% of cells were protoplasmic (i.e. non-process-bearing) and 28% fibrous (process-bearing) cells with 8% of cells visible under phase contrast not stained positive with GFAP antiserum. Ninety-four per cent of cells were positively stained with GFAP antiserum in the protoplasmic cultures.

Accumulation of [^{3}H]*cyclic AMP.* Cyclic AMP accumulation was measured using a modification of the method described previously for brain slices [25]. Cells were incubated for 2 hr at 37° in 1 mL/well of DMEM 10% FCS containing 0.08 μM (2 μCi/well) 8-[^{3}H]adenine in a water saturated atmosphere of 10% CO_2 in air. Prelabelled cell monolayers were then washed three times with 1 mL/well DMEM alone and incubated for 15 min in 1 mL/well DMEM at 37°. Agonists were then added in 10 μL of DMEM and the incubation continued for a further 10 min. Where appropriate, antagonists were equilibrated with the cells for 30 min prior to agonist addition. Incubations were terminated by the addition of 50 μL of 10 M HCl. [^{3}H]Cyclic AMP was then isolated by sequential Dowex–alumina chromatography as previously described [25]. Prior to elution, samples were spiked with [^{14}C]cyclic AMP to allow for percentage recovery correction. Eluates were collected, 300 μL of 1 M HCl and scintillant were added and the levels of [^{3}H]- and [^{14}C]cyclic AMP determined by liquid scintillation counting.

Data analysis. Agonist concentration–response curves were drawn by inspection or fitted to a logistic equation using the non-linear regression program GraphPAD (ISI). The equation fitted was:

$$\text{Response} = \frac{E_{max} \times X^n}{(EC_{50})^n + X^n} + B$$

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

Unless otherwise stated, all values given in the text represent mean ± SE of N separate experiments. Statistical analysis was performed using the Student's t-test.

Materials. 8-[^{3}H]Adenine (26 Ci/mmol) was purchased from Amersham International (Aylesbury, U.K.) and 8-[^{14}C]cyclic adenosine monophosphate (45 mCi/mmol) ammonium salt was obtained from New England Nuclear (Stevenage, U.K.). Noradrenaline, isoprenaline, atenolol HCl, phentolamine, 3-isobutyl-1-methylxanthine and yohimbine were all supplied by the Sigma Chemical Co. (Poole, U.K.). The intermediate filament protein antibodies to glial fibrillary acidic protein and vimentin and the corresponding fluorescent antibodies were purchased from Dako patt (Denmark). Dulbecco's modification of Eagles medium (DMEM) and the foetal bovine serum were supplied by Northumbria Biologicals (Northumberland, U.K.). The trypsin EDTA solution was purchased from Gibco (Uxbridge, U.K.). Prazosin was a gift from Ciba-Geigy (Basel, Switzerland). Isradipine (PN200110) was supplied by Sandoz (Basel, Switzerland). Tissue culture plastics including 75 cm flasks and 24-well cluster dishes were obtained from Costar (Northumbria Biologicals). The rats were of the Wistar strain and were obtained from the Medical School Animal Unit, University of Nottingham.

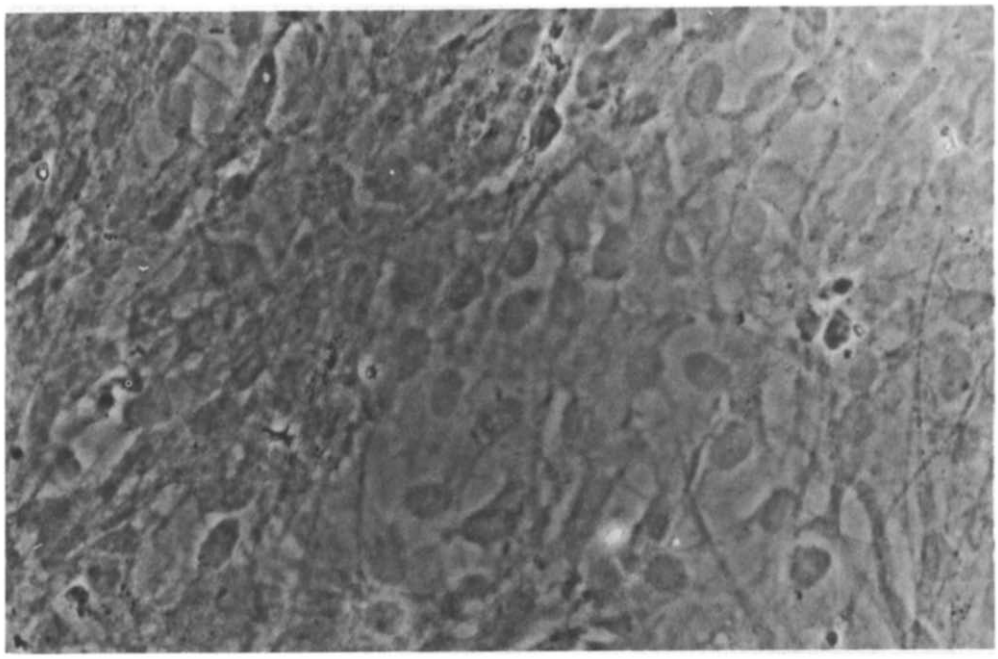
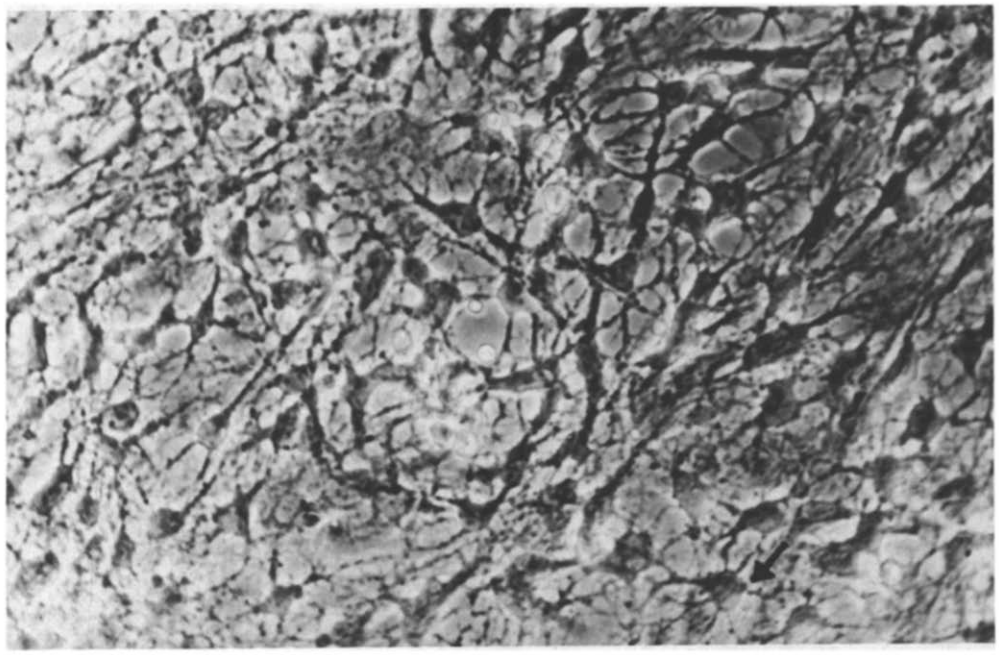


Fig. 1(a).



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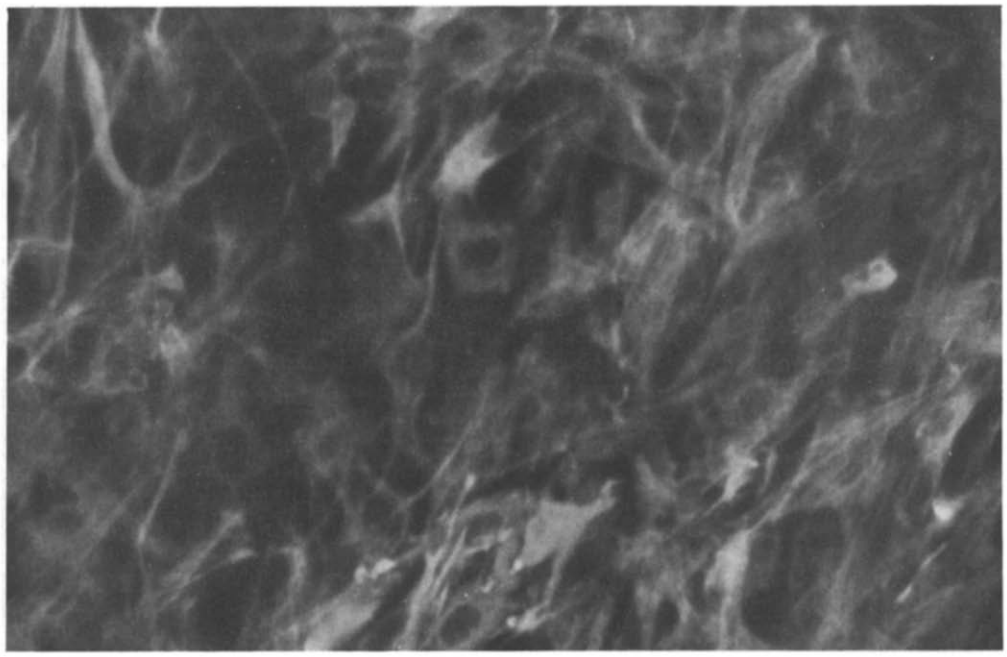
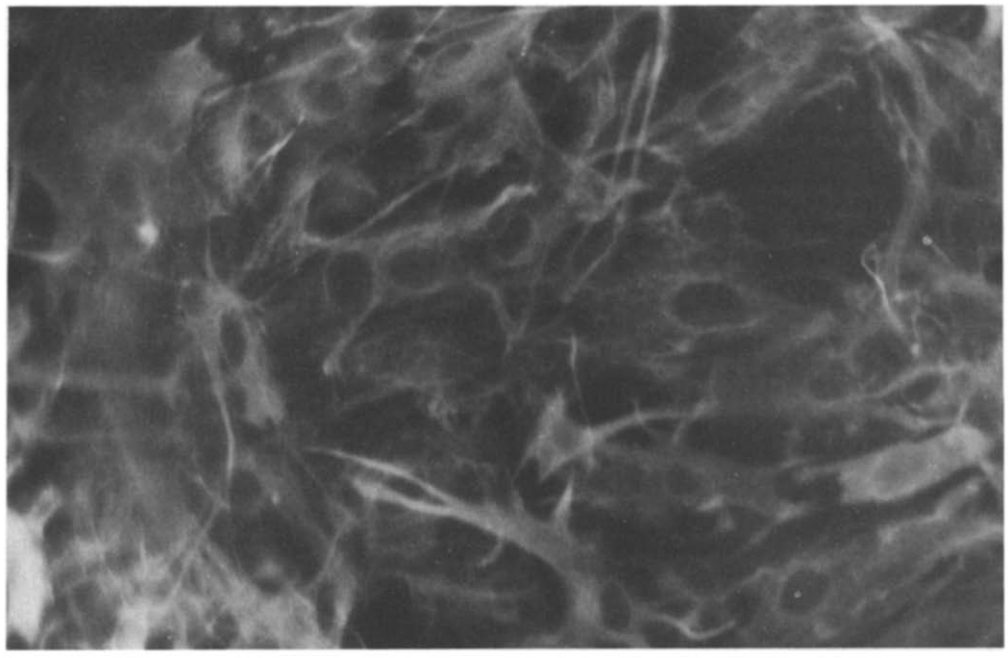


Fig. 1(c).



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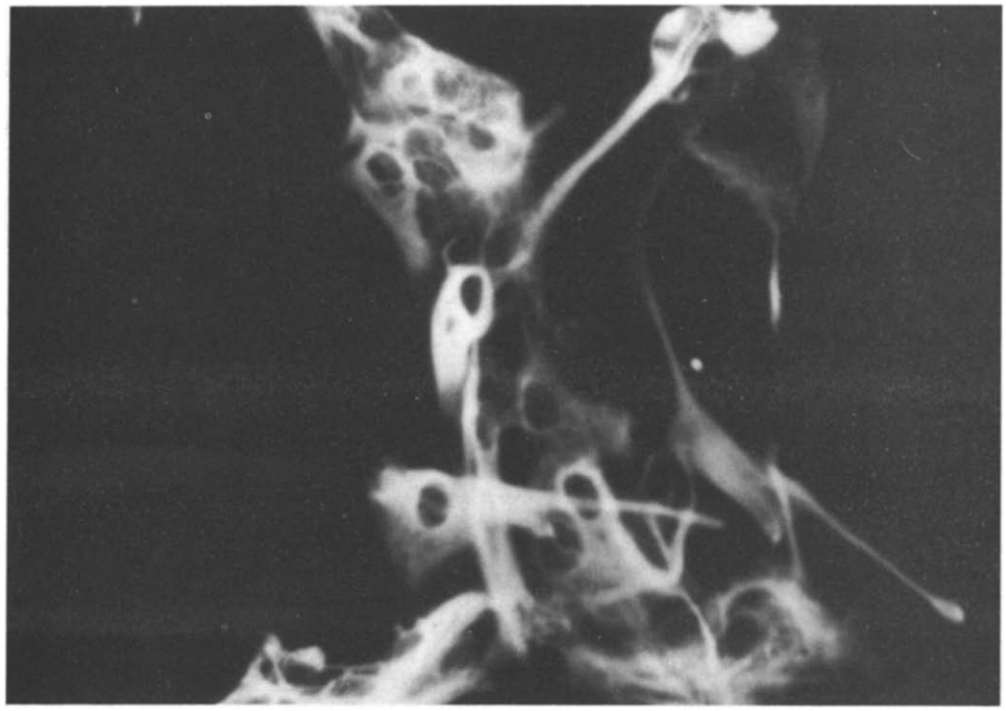

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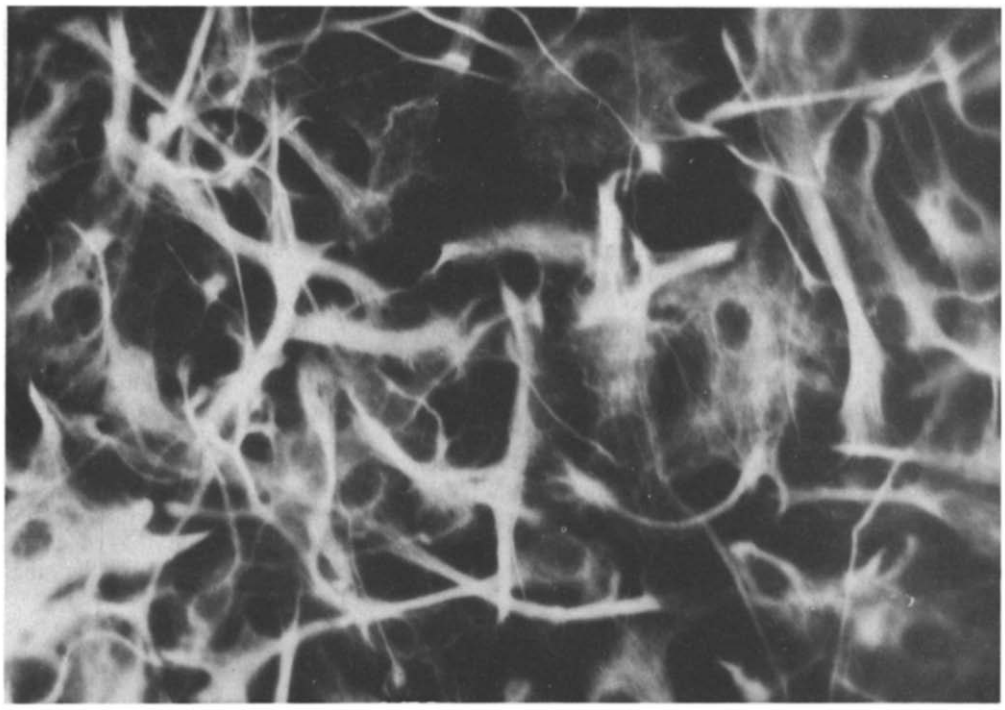

Fig. 1(f).

Fig. 1. Purified protoplasmic (8-day-old animals) and fibrous/protoplasmic (2-day-old animals) astroglia were prepared as described in Materials and Methods and replated onto glass coverslips 7 days prior to staining with intermediate filament protein antiserum. (a) Phase contrast protoplasmic astroglia. (b) Phase contrast fibrous/protoplasmic astroglia. The arrow indicates the morphology of the fibrous cells. (c) Protoplasmic astroglia stained with vimentin antiserum. (d) Fibrous/protoplasmic astroglia stained with vimentin antiserum. (e) Protoplasmic astroglia stained with GFAP antiserum. (f) Fibrous/protoplasmic astroglia stained with GFAP antiserum.

RESULTS

Identification of cell types

Figures 1a and b show the phase contrast micrographs of cultures prepared from 8-day-old rat brain (protoplasmic) and 2-day-old rat brain (fibrous/protoplasmic). The protoplasmic astrocytes can be seen as flat polygonal shaped cells (Fig. 1a) which make up the bed layer in the mixed protoplasmic/fibrous culture (Fig. 1b). The process-bearing fibrous astroglia can be seen growing on top of the protoplasmic astroglia (Fig. 1b, arrow). Immunofluorescence studies using the antibodies to glial fibrillary acidic protein (GFAP) and vimentin showed that cultures containing protoplasmic cells alone stained positively for vimentin (Fig. 1c). Diffuse staining of the intermediate filaments can clearly be seen around large, relatively unstained, nuclei. The underlying protoplasmic cells of the mixed cultures (Fig. 1d) similarly stained positively for vimentin with little staining of the overlying fibrous cells. This is consistent with the finding that immature (protoplasmic) astrocytes express more vimentin than mature (fibrous) astrocytes (Fig. 1c and d). The GFAP-stained filaments of the protoplasmic cells (Fig. 1e) had their usual fibrillary appearance around a large, relatively less stained nucleus (Fig. 1e). This "fried egg" appearance of the GFAP staining was also evident in the underlying layer of protoplasmic cells in the mixed cultures (Fig. 1f). In the mixed cultures, however, intense staining of the overlying process-bearing fibrous cells with GFAP can clearly be seen (Fig. 1f). Non immune serum did not reveal any appreciable staining of the glial cell cultures.

Pharmacology of the adrenoceptor-mediated cyclic AMP response in primary astroglia

Protoplasmic cells. Figure 2a shows the composite concentration–response curves for isoprenaline and noradrenaline stimulated cyclic AMP accumulation in the protoplasmic (8-day-old rat brain) astroglial cell cultures. Both isoprenaline and noradrenaline exhibited a maximal response at a concentration of 10 μM. The EC_{50} values for the two catecholamines were 0.05 and 0.5 μM, respectively (Table 1). The maximum response to isoprenaline represented a stimulation of 24-fold over basal levels (N = 5) whilst that for noradrenaline represented *ca.* 50% of that obtained with isoprenaline (Fig. 2a). A combination of the two catecholamines noradrenaline (1 or 10 μM) and isoprenaline (1 μM) produced a cyclic AMP response which was 58.0 ± 7.2% and 64.0 ± 12.4% of that produced by isoprenaline alone (Fig. 3; Table 2). This attenuation of the response

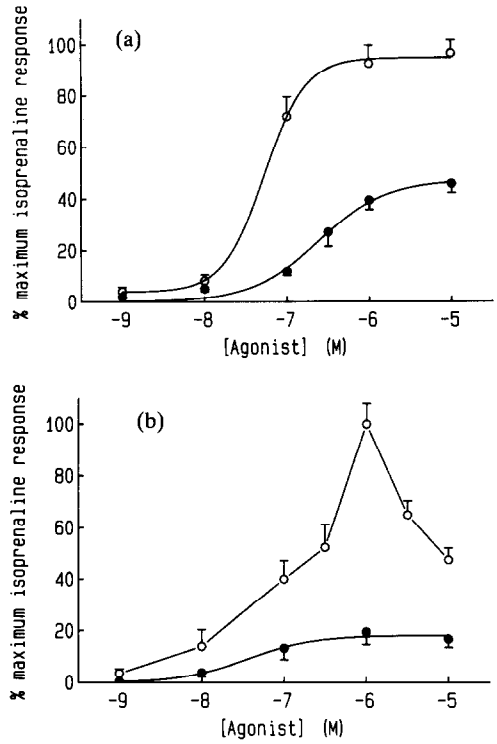

Fig. 2. Concentration–response curves for isoprenaline (○) and noradrenaline (●) induced [^{3}H]cyclic AMP accumulation in protoplasmic (a) and fibrous/protoplasmic (b) astroglial cell cultures. Values represent combined mean ± SE of quadruplicate determinations of 4 (a) or 7 (b) separate experiments. Data for noradrenaline and isoprenaline in protoplasmic or fibrous/protoplasmic cells were obtained from the same cell preparation.

to isoprenaline by noradrenaline was antagonized by the α_2-adrenoceptor antagonist yohimbine (1 μM) (Table 2; Fig. 3).

Mixed fibrous/protoplasmic cultures. Figure 2b shows the composite dose–response curves for isoprenaline and noradrenaline stimulated cyclic AMP accumulation in co-cultures of fibrous/protoplasmic astroglia. The EC_{50} values for isoprenaline and noradrenaline were 0.8 and 0.17 μM, respectively (Table 1). The maximum accumulation of cyclic AMP produced by isoprenaline (11-fold over basal levels; N = 5) was lower than that obtained in protoplasmic cultures and the concentration–response curve was much flatter over the range 10^{-9} to 10^{-6} M. Furthermore, the relative size of the response to noradrenaline (with respect to

Table 1. Agonist EC_{50} values in protoplasmic and fibrous/protoplasmic astroglia

Agonist	Protoplasmic EC_{50} (μM)	Fibrous/protoplasmic EC_{50} (μM)
Isoprenaline	0.051 ± 0.01 (5)	0.80 ± 0.09 (5)*
Noradrenaline	0.47 ± 0.12 (5)	0.17 ± 0.03 (5)†

Values represent mean ± SE. Numbers in parentheses refers to the number of experiments performed.

* $P < 0.001$ or † $P < 0.05$ with respect to value in protoplasmic cultures.

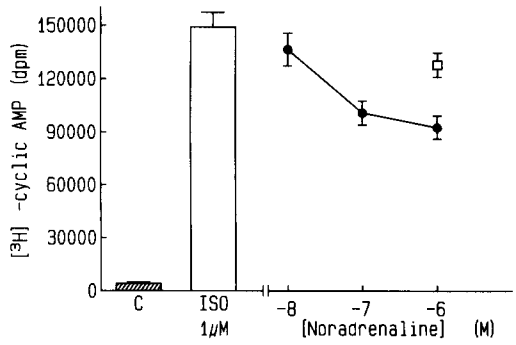

Fig. 3. Effect of increasing concentrations of noradrenaline on the cyclic AMP response to 1 μM isoprenaline in protoplasmic astroglia. The basal accumulation of [^{3}H]cyclic AMP is shown by the histogram marked C and the response to 1 μM isoprenaline alone is shown by the histogram marked ISO. The open square shows the effect of 1 μM yohimbine on the response to a combination of isoprenaline (1 μM) and noradrenaline (1 μM). Values represent mean ± SE of quadruplicate determinations in a single experiment. Similar data were obtained in two other experiments.

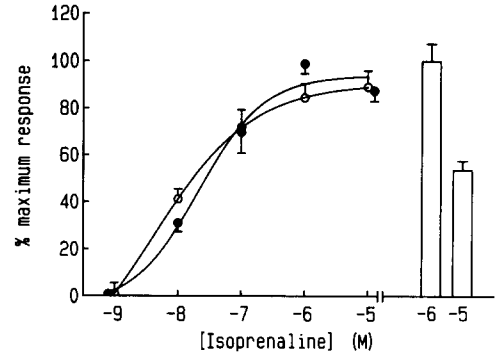

Fig. 4. Effect of α-adrenoceptor antagonists on the isoprenaline concentration–response curve in fibrous/protoplasmic astroglia. Antagonists were added 30 min prior to agonist administration. Data are expressed as a percentage of the response to 1 μM isoprenaline in the absence of antagonists, obtained after subtraction of the basal levels of [^{3}H]cyclic AMP accumulation. The histograms show the control responses to 1 and 10 μM isoprenaline. Data represent mean ± SE of quadruplicate determinations in each of three separate experiments. (○) Phentolamine (1 μM); (●) prazosin (1 μM). The response to 10 μM isoprenaline was significantly less ($P < 0.05$) than that to 1 μM isoprenaline in the absence of α-adrenoceptor antagonists.

isoprenaline) was substantially reduced compared to pure protoplasmic cultures (Fig. 2). A striking feature of the concentration–response curve for isoprenaline was that the cyclic AMP response decreased sharply at concentrations above 1 μM (Fig. 2b). This phenomenon was not seen in cultures containing protoplasmic astroglia alone (Fig. 2a). A combination of isoprenaline (1 μM) and noradrenaline (1 μM, 10 μM) produced a cyclic AMP response which was only 26 ± 2% and 38 ± 4% respectively, of that produced by isoprenaline alone (Table 2). This attenuation of the response to isoprenaline by noradrenaline was antagonized to a large extent by the α-adrenoceptor antagonist yohimbine (Table 2). Similarly, the response to 1 μM noradrenaline alone in these cells was augmented by 1 μM yohimbine (from 22 ± 7% to 70 ± 9% of the response to 1 μM isoprenaline) but not by the α_1-adrenoceptor antagonist prazosin (1 μM; N = 3; data not shown).

Effects of α-adrenoceptor antagonists on the response to isoprenaline in fibrous/protoplasmic astroglia

It has been reported previously that the fall in the isoprenaline dose–response curve at high concentrations of isoprenaline in mouse astrocytes could be reversed by the α-adrenoceptor antagonist phentolamine [8]. Figure 4 shows the concentration–response curve to isoprenaline obtained in the presence of two α-adrenoceptor antagonists in fibrous/protoplasmic astroglia co-cultures. As reported previously in the mouse, the fall in the isoprenaline concentration–response curve was not observed in rat astrocytes in the presence of 1 μM phentolamine. Surprisingly, this effect of phentolamine was mimicked by the α_1-adrenoceptor antagonist prazosin (1 μM; Fig. 4) but not by the

Table 2. Effect of noradrenaline on the isoprenaline stimulated β-adrenoceptor response in two cultures of astroglial cells

Agonist	% Maximum response to isoprenaline	
	Protoplasmic	Fibrous/protoplasmic
Isoprenaline (1 μM)		
+noradrenaline (1 μM)	58.0 ± 7.2 (9)†	26.0 ± 2.1 (5)†
Isoprenaline (1 μM)		
+noradrenaline (10 μM)	63.6 ± 12.4 (5)*	38.0 ± 4.0 (3)*
Isoprenaline (1 μM)		
+noradrenaline (1 μM)		
+yohimbine (1 μM)	80.0 ± 3.4 (4)‡	85.4 ± 8.0 (5)§
Isoprenaline (1 μM)		
+yohimbine (1 μM)	ND	97.3 ± 5.9 (4)

Values represent mean ± SE. The number of separate experiments is given in parentheses. Yohimbine was added 30 min prior to the addition of agonist.

* $P < 0.05$, or † $P < 0.001$ with respect to maximum response to isoprenaline.

‡ $P < 0.05$ or § $P < 0.001$ with respect to responses in the absence of yohimbine.

ND, not determined.

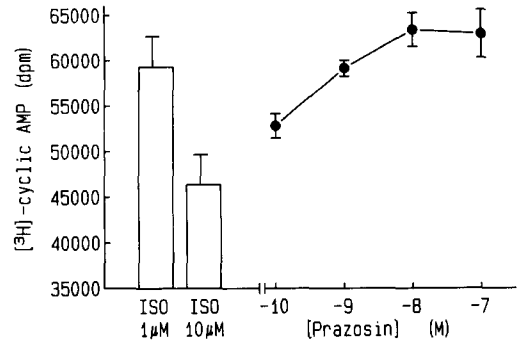

Fig. 5. Effect of prazosin on the cyclic AMP response to 10 μM isoprenaline in fibrous/protoplasmic astroglia. (●) Responses to 10 μM isoprenaline in the presence of increasing concentrations of prazosin. Prazosin were added 30 min prior to agonist administration. The histograms show the control responses to 1 and 10 μM isoprenaline in the absence of antagonist which were significantly different ($P < 0.05$). Data represent mean ± SE of quadruplicate determinations in a single experiment. Similar data were obtained in a second experiment.

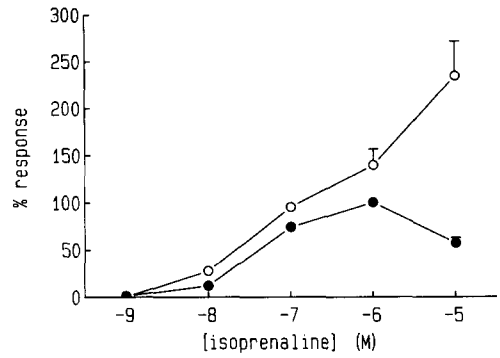

Fig. 7. The effect of IBMX on the concentration–response curve to isoprenaline in mixed fibrous/protoplasmic astroglial cultures. IBMX was added 30 min prior to agonist addition. (●) Control; (○) IBMX (0.1 mM). Data are expressed as a percentage of the response to 1 μM isoprenaline in the absence of IBMX, obtained after subtraction of the basal levels of [^{3}H]cyclic AMP accumulation. Data represent mean ± SE of quadruplicate determinations in each of three separate experiments.

α_2-antagonist yohimbine (1 μM; data not shown; N = 2). Prazosin was able to antagonize the inhibitory response to 10 μM isoprenaline in a concentration-dependent manner with an IC_{50} of 0.5 nM (Fig. 5).

Effect of calcium free incubation conditions and isradipine

To establish whether calcium influx could contribute to the inhibitory effect of high concentrations of isoprenaline on cyclic AMP accumulation in mixed fibrous/protoplasmic astroglial cultures, we have performed experiments in the presence of calcium-free media or the dihydropyridine calcium channel antagonist isradipine [26]. Both interventions restored the isoprenaline log concentration–response curve to a sigmoidal relationship, although the curve obtained in the presence of isradipine was rather steeper (Fig. 6).

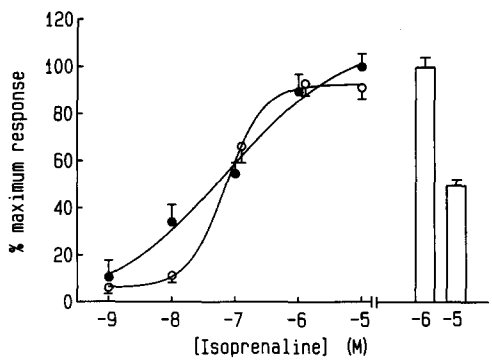

Fig. 6. Effect of Ca^{2+}-free incubation conditions and isradipine on the isoprenaline concentration–response curve in fibrous/protoplasmic astroglia. Measurement of cyclic AMP accumulation was measured in Hanks balanced salts (± calcium) buffered with 20 mM Hepes. Incubations were performed at 37° in air. Isradipine (10 μM) was added 30 min before the addition of agonist. Data are expressed as a percentage of the response to 1 μM isoprenaline in the absence of isradipine in normal calcium-containing media, obtained in each experiment after the subtraction of the basal levels of [^{3}H]cyclic AMP accumulation. The histograms show the control responses to 1 and 10 μM isoprenaline in calcium-containing media which were significantly different ($P < 0.05$). Data represent a combined mean ± SE of quadruplicate determinations obtained in each of three different experiments. Similar data were obtained with isradipine using DMEM. (○) Isradipine; (●) Ca^{2+} free incubation conditions.

Effect of 3-isobutyl-1-methylxanthine

In astrocytoma cells and FRTL5 cells, a reduction in cellular cyclic AMP levels can be elicited by an

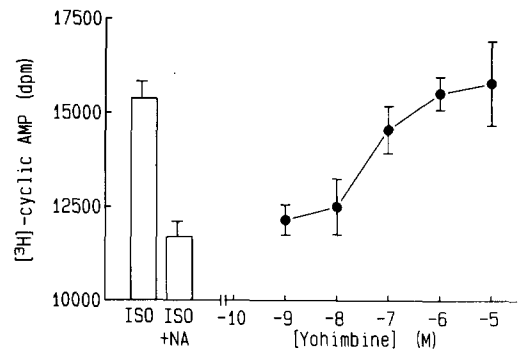

Fig. 8. Effect of yohimbine on the cyclic AMP response to a combination of isoprenaline and noradrenaline in fibrous/protoplasmic astroglia in the presence of 0.1 mM IBMX. (●) Responses to 1 μM isoprenaline + 1 μM noradrenaline in the presence of increasing concentrations of yohimbine. Yohimbine and IBMX were added 30 min prior to agonist administration. The histograms show the control responses to 1 μM isoprenaline (ISO) and 1 μM isoprenaline + 1 μM noradrenaline (ISO + NA) in the absence of antagonist. IBMX was present in every incubation. Data represent mean ± SE of quadruplicate determinations in a single experiment. The responses to isoprenaline + noradrenaline in the absence ($P < 0.001$) and presence of 10^{-9} M ($P < 0.001$) or 10^{-8} M yohimbine ($P < 0.05$) were significantly less than the control isoprenaline response. Similar data were obtained in a second experiment.

influx of extracellular calcium due to an activation of a calcium-dependent phosphodiesterase enzyme activity [27, 28]. To determine whether a similar mechanism was operating in mixed fibrous/protoplasmic astroglial cultures, concentration–response curves to isoprenaline were obtained in the presence and absence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM; Fig. 7). In the presence of IBMX, the observed fall in response of the control curve at high agonist concentrations was replaced by a further stimulation of cyclic AMP accumulation above that produced by 1 μM isoprenaline (Fig. 7). Although IBMX amplified the cyclic AMP response to all isoprenaline concentrations, there was no significant effect on the basal [^{3}H]cyclic AMP levels (N = 4). In the presence of IBMX (0.1 mM), noradrenaline (1 μM) was still able to inhibit the response to 1 μM isoprenaline and this effect could be reversed by the α_2-adrenoceptor antagonist yohimbine (IC_{50} = 40 nM; Fig. 8).

DISCUSSION

The present study has demonstrated the presence of both α- and β-adrenoceptors modulating cyclic AMP accumulation in astroglial cell cultures containing either protoplasmic cells alone or a mixed population of fibrous and protoplasmic astroglia. These findings are in broad agreement with those obtained in previous studies where astrocytes were prepared from 2-day-old animals and presumably contained a mixture of fibrous and protoplasmic cells [7–9]. In both cell systems, β-adrenoceptor stimulation increased cyclic AMP levels while α-adrenoceptor activation (with noradrenaline) attenuated the cyclic AMP response to isoprenaline. This inhibitory response to noradrenaline appeared to be mediated by α_2-adrenoceptors in both cell culture systems since it could be antagonized by the α_2-antagonist yohimbine but not by the α_1-selective antagonist prazosin.

There were, however, differences in both the shape of the concentration–response curve to isoprenaline and the extent of the α_2-adrenoceptor-mediated inhibition of cyclic AMP accumulation between the two astroglial cell preparations. The concentration–response curve to isoprenaline in the mixed fibrous/protoplasmic cultures was characterized by a fall in cyclic AMP levels at concentrations above 1 μM isoprenaline which was not evident in cultures containing only protoplasmic cells.

Previous studies in mouse astrocytes have shown that this reduction in response at high concentrations of isoprenaline can be inhibited by the α-adrenoceptor antagonist phentolamine [8]. Van Calker *et al.* [8] have therefore suggested that high concentrations of isoprenaline can mediate a depressant effect on cyclic AMP accumulation via the activation of α_2-adrenoceptors. In view of the apparently greater α_2-inhibitory response in the mixed fibrous/protoplasmic cultures, this suggestion could partly explain why the concentration–response curve in protoplasmic cultures was much better maintained at high concentrations of isoprenaline in the present study.

Although the results of the present study confirm that phentolamine can attenuate the fall in cyclic AMP levels at high concentrations of isoprenaline in rat astrocytes, they suggest that this is due to α_1-, rather than α_2-, adrenoceptor antagonism. Thus, the α_1-adrenoceptor antagonist prazosin (IC_{50} = 0.5 nM) was able to mimic the effect of phentolamine (1 μM) at concentrations selective for the α_1-adrenoceptor, whereas the α_2-adrenoceptor antagonist yohimbine failed to prevent the marked reduction in cyclic AMP levels, observed at 10 μM isoprenaline, at concentrations up to 1 μM.

Since the affinity of noradrenaline is higher for α_2-compared with α_1-adrenoceptors (at least as measured by radioligand binding to rat brain membranes) it is not surprising perhaps that the inhibitory effect of 1 μM noradrenaline on the isoprenaline response in mixed fibrous/protoplasmic cultures was largely reversed by yohimbine with prazosin having little or no effect (Table 2). A stimulation of α_1-adrenoceptors would only be expected at higher concentrations of noradrenaline. The inhibitory effect of isoprenaline at concentrations of the order of 10 μM is, however, surprising given that, on the basis of data obtained from studies using rat cortical membranes, isoprenaline would not be expected to occupy a significant number of α_1-adrenoceptors at these concentrations. However, the data obtained with prazosin clearly demonstrate an involvement of α_1-receptors in the response to isoprenaline and indicate that, unlike the situation with noradrenaline, this is not masked by an α_2-adrenoceptor-mediated attenuation of adenylate cyclase activity.

In the presence of the calcium channel antagonist isradipine or under nominally calcium-free conditions, the fall in the isoprenaline concentration-response curve was also prevented. In hippocampal neurons, isoprenaline has been shown to open voltage dependent calcium channels [29] by stimulating cyclic AMP accumulation via β-adrenoceptors, but given the reversal of the calcium dependent inhibitions by prazosin and phentolamine it is unlikely that such a mechanism is involved in these astrocytes.

Taken together these data suggest that, at high concentrations, isoprenaline exerts a negative effect on cyclic AMP levels through an α_1-adrenoceptor linked directly, or indirectly, to the opening of a dihydropyridine-sensitive calcium channel. The resulting influx of calcium may then reduce cyclic AMP levels by activating a calcium-dependent phosphodiesterase enzyme such as that observed in astrocytoma cells and FRTL5 cells [27, 28]. Support for this hypothesis is provided by the observation (Fig. 7) that inhibition of Ca^{2+}-stimulated PDE activity with the non-selective PDE-inhibitor IBMX prevented any reduction in cyclic AMP accumulation at high concentrations of isoprenaline. Indeed, the fact that cyclic AMP levels were further increased at 10 μM isoprenaline (over those obtained with 1 μM agonist), in the presence of IBMX, indicates that the extent of PDE activation may be underestimated, in some experiments, when reductions in cyclic AMP levels are measured relative to those obtained in the presence of 1 μM isoprenaline.

The existence of an α_1-adrenoceptor subtype (α_{1a})

directly coupled to dihydropyridine-sensitive calcium channels in smooth muscle has been reported [30] and since some selective antagonists for this receptor are now available it will be of interest to determine their effects on the inhibitory phase of the isoprenaline response in future experiments. Interestingly, α_1-adrenoceptors mediating inositol phospholipid hydrolysis have been detected in newborn rat primary astrocyte cultures (which presumably contain both astroglial cell types) [19] and α_1-adrenoceptor-mediated depolarizations have been observed in immunohistologically-defined fibrous cells [31].

The difference in the characteristics of the isoprenaline dose–response curves in the two cell culture preparations indicates that this α_1-adrenoceptor modulation may only be present on the fibrous astrocytes, which make up 28% of the cells in the mixed fibrous/protoplasmic cultures. However, a striking observation made by McCarthy and co-workers [14, 32, 33] using combined immunofluorescent and autoradiographic techniques was that although protoplasmic astroglia possess substantial numbers of β-adrenoceptors, fibrous astroglia express less than 5% of the β-adrenoceptor number detected in protoplasmic cells. This raises the intriguing possibility that there may be some form of "cross-talk" between the two astroglial cell types with the inhibitory effect of α_1-receptor stimulation on β-adrenoceptor-stimulated cyclic AMP being mediated by the calcium-dependent release and action of some inhibitory factor(s). The increased susceptibility of the isoprenaline cyclic AMP response in mixed cultures to inhibition by α_2-adrenoceptor activation could also be partly due to such an interaction, although the fact that α_2-adrenoceptor-mediated inhibitory responses can still be demonstrated in the presence of IBMX (Fig. 8) indicates that the actual mechanisms involved are quite separate. Alternatively, the presence of fibrous astroglia may alter the expression of α_2-adrenoceptors in the protoplasmic cells during cell culture.

The slope of the concentration–response curve to isoprenaline in mixed fibrous/protoplasmic cultures, which is clearer to picture in the presence of IBMX (Fig. 7), is markedly flatter than that obtained in pure protoplasmic cultures (Fig. 1). This observation, taken together with the markedly different apparent EC_{50} values (Table 1) suggests that the concentration–response curve in the mixed astroglial cultures may contain two components; one of which may have an EC_{50} close to that obtained in pure protoplasmic cultures (cf. Figs 1 and 7). This, however, might not be totally unexpected in a mixed cell population containing cells with markedly different degrees of β-adrenoceptor expression [14, 32, 33]. It remains a possibility, therefore, that because of the low density of β-adrenoceptors on fibrous cells, isoprenaline only begins to raise cyclic AMP levels in this portion (i.e. the fibrous cells) of mixed astroglial cultures at high concentrations which are close to the range at which α_1-adrenoceptor-mediated inhibition of cyclic AMP accumulation becomes apparent.

The ages of the animals used to prepare the two cultures of astrocytes used in this study may also play a significant role in the expression and/or interaction of α- and β-adrenoceptors. It has been reported that fibroblast contamination increases with increasing age of the animal used for astroglial culture and that cultures prepared from neonatal animals contain contaminating oligodendroglia [7]. Since both fibroblasts and oligodendroglia contain β-adrenoceptors they could contribute to the β-adrenoceptor response seen in the culture systems used in this study. To remove contaminating cells both protoplasmic and mixed fibrous/protoplasmic astroglial cell cultures were subjected to orbital shaking [21]. No differences in the cyclic AMP responses were observed either with or without shaking. It would seem therefore that the differences in the isoprenaline concentration–response curve and the extent of the α-adrenoceptor mediated inhibition of the isoprenaline response observed in this study is due to the presence of fibrous astrocytes in one of the culture systems (2-day-old preparation) and not in the other (8-day-old preparation).

In conclusion, the data from this study suggest that two different populations of astrocytes in *in vitro* culture are able to raise intracellular cyclic AMP levels via β-adrenoceptor activation and that there are differences in the extent of α-adrenoceptor mediated inhibition of cyclic AMP accumulation between the two primary astroglial cell preparations. It remains to be established, however, whether this is due to a preferential expression of α-adrenoceptors (both α_1 and α_2) in the fibrous astroglia or to an interaction between the two different cell classes.

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