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Agonists Calcitonin, Corticotropin-Releasing Hormone, and Vasoactive Intestinal Peptide, but Not Prostaglandins or β-Adrenergic Agonists, Elevate Cyclic Adenosine Monophosphate Levels in Oligodendroglial Cells

Anthony P. Wiemelt,[1,2] **Maria Lehtinen,**[1] **and F. Arthur McMorris**[1,2]*
[1]The Wistar Institute, Philadelphia, Pennsylvania
[2]Graduate Group in Neuroscience, University of Pennsylvania, Philadelphia

Although 3′,5′-cyclic AMP (cAMP) is known to regulate oligodendrocyte development in vitro, little is known about the identity of agonists that induce cAMP synthesis in oligodendroglia. To identify such agonists, we used a novel immunohistochemical method of visualizing cAMP within single cells to screen compounds that are known to activate cAMP synthesis in other cellular systems. Calcitonin, corticotropin-releasing hormone, and vasoactive intestinal peptide elevated cAMP in oligodendroglial cells but not in other cell types present in the cultures (i.e., astrocytes and microglia). In contrast, prostaglandins and the β-adrenergic agonist isoproterenol, which have previously been reported to induce modest increases in oligodendroglial cell cAMP from biochemical assay of cell homogenates, did not induce a detectable cAMP response in individually identified oligodendroglial cells but instead induced a robust cAMP response in nonoligodendroglial cells. J. Neurosci. Res. 65:165–172, 2001. © 2001 Wiley-Liss, Inc.

Key words: oligodendrocyte; astrocyte; microglia; signal transduction

INTRODUCTION

3′,5′-Cyclic AMP (cAMP) regulates many aspects of oligodendrocyte development. Elevation of cAMP accelerates the rate at which mature oligodendrocytes appear in primary glial cell cultures and induces the expression of the myelin proteins 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), proteolipid protein, and myelin basic protein (McMorris, 1983a; Raible and McMorris, 1989, 1990; Jensen et al., 1993). By a separate mechanism, cAMP inhibits the proliferation of oligodendroglial precursors (McMorris, 1983b; Raible and McMorris, 1993). More recently we have shown that, even at unstimulated (basal) levels, cAMP is required for morphological differentiation and process outgrowth of oligodendrocytes (Wiemelt and McMorris, submitted). However, although cAMP exerts such potent and varied regulatory effects in developing oligodendroglia, little is known about the biological agonists that activate cAMP production within oligodendroglial cells.

Several previous studies have reported agonists that produce modest elevations in cAMP from homogenates of oligodendroglial cell cultures (McCarthy and de Vellis, 1980; Vartanian et al., 1988; Cohen and Almazan, 1994; Ghiani et al., 1999). However, biochemical measurements of cAMP from cell homogenates are of limited value in evaluating the ability of agonists to stimulate cAMP production within oligodendroglia. Even when enriched for oligodendroglia, primary glial cell cultures almost always contain a small percentage of contaminating cells, such as astrocytes and microglia, and usually consist of oligodendroglia at different stages of differentiation (McCarthy and de Vellis, 1980; Behar et al., 1988; Vartanian et al., 1988; Cohen and Almazan, 1994; A.P.W. and F.A.M., unpublished observations). Prior to the present study, no agonist has been shown to elevate cAMP in a cell identified directly as an oligodendroglial cell.

Having demonstrated that oligodendroglia elevate endogenous cAMP levels in response to activation of adenylate cyclase by forskolin and cholera toxin (Wiemelt

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Maria Lehtinen's current address is: Department of Neurobiology, Harvard Medical School, Boston, MA 02115.

Anthony P. Wiemelt's current address is: Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104.

*Correspondence to: F. Arthur McMorris, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104.
E-mail: mcmorris@mail.wistar.upenn.edu

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et al., 1997; Wiemelt and McMorris, submitted), we desired to identify ligands that can stimulate cAMP production in these cells via binding to cell-surface receptors. We tested agonists that have been previously reported to elevate cAMP in oligodendroglial cell cultures, as well as agonists that have been shown to stimulate the production of cAMP in other cell types via $G\alpha_s$-containing G-protein-coupled receptors (GPCRs), for their ability to increase cAMP in individually identified oligodendroglial cells. We report here that calcitonin, corticotropin-releasing hormone (CRH), and vasoactive intestinal peptide (VIP) elevate cAMP within individually identified oligodendroglial cells, whereas prostaglandin E1 (PGE1) and the β-adrenergic agonist isoproterenol do not.

MATERIALS AND METHODS

Reagents

Forskolin and rolipram were obtained from Calbiochem (San Diego, CA); cholera toxin and 3-isobutyl-1-methylxanthine (IBMX) were obtained from RBI (Natick, MA); acrolein and $NaCNBH_3$ were obtained from Sigma (St. Louis, MO). See Table I for a summary of agonists used and their sources.

Preparation of Primary Glial Cell Cultures

Primary glial cell cultures were isolated from dissociated postnatal day 1 rat cerebra as described elsewhere (McCarthy and de Vellis, 1980; McMorris et al., 1986). Such cultures contain a mixture of glial cells, including oligodendrocytes, astrocytes, and microglial cells. Cultures were grown in oligodendrocyte medium 5 (OM-5; Raible and McMorris, 1990) at 37°C for 5 days until postnatal day 6 and were then enriched for oligodendroglia by differential shake-off as described elsewhere (McCarthy and de Vellis, 1980; McMorris et al., 1986). On postnatal day 7, cells were transferred into 150 mm bacterial Petri dishes for 30 min at 37°C, during which time astrocytes and microglia adhere preferentially to the surface, while oligodendroglia remain in suspension (Liu and Almazan, 1995). Cells remaining in suspension were gently recovered from these plates and inoculated onto 16-well polylysine-coated culture slides in OM-5 medium for an additional 1 day at 37°C. Treatments were initiated the following day (postnatal day 8) in oligodendrocyte medium 6 (OM-6; Raible and McMorris, 1990) containing 1% fetal bovine serum and 5 μg/ml insulin (referred to hereafter as OM-6+).

Agonist Treatment

To screen GPCR agonists for their ability to stimulate cAMP production in oligodendroglia, we tested agonists at concentrations and lengths of treatment shown to be effective in other cell types, as reported in the literature (see Table I). On postnatal day 8, mixed glial cultures were switched to OM-6+ with or without platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2; both at 5 ng/ml) for 48 hr. On postnatal day 10, cultures were pretreated for 30 min with IBMX (1 mM) and rolipram (80 μM) to block cAMP phosphodiesterase activity, and then individual agonists or agonist/antagonist combinations were added at the concentrations indicated in the text and in Table I for an additional 45 min.

Immunofluorescence Staining

Oligodendroglial markers. Monoclonal antibodies recognizing antigens expressed at different oligodendroglial developmental stages were used as tissue culture supernatants from hybridoma clones. Monoclonal antibody A2B5 (Eisenbarth et al., 1979) was used to distinguish early oligodendroglial precursor cells; antibody O4 (Sommer and Schachner, 1981) was used to identify intermediate precursors; O1 (Sommer and Schachner, 1981) was used to identify oligodendrocytes. Optimal staining of surface antigens was achieved by incubating live cultures with antibody for 30 min prior to fixation with acrolein (see below). Surface antibodies were then detected with secondary antibody conjugated directly to Texas red (1:100; Vector, Burlingame, CA). Live staining with marker antibodies does not change cAMP content of the cells (Wiemelt et al., 1997).

cAMP immunofluorescence. cAMP immunostaining was performed as described previously (Wiemelt et al., 1997). Briefly, cells were fixed with 5.5% acrolein, washed, and incubated for 30 min with affinity-purified rabbit anti-cAMP antiserum at 1:50 dilution. Bound antibody was detected with biotinylated goat anti-rabbit secondary antibody (1:200; Vector) and fluorescein-coupled avidin (1:100; Vector). Confocal immunofluorescence images were obtained using a Leica confocal microscopy unit. Details of fixation, antibody staining, and confocal microscopy are given by Wiemelt et al. (1997).

RESULTS

Cultures were exposed to various agonists in the presence of the cAMP phosphodiesterase (PDE) inhibitors IBMX and rolipram; immunostained with a mixture of monoclonal antibodies A2B5, O4, and O1 to detect oligodendroglial cells; fixed; and immunostained with affinity-purified anti-cAMP antiserum to detect cAMP (see Materials and Methods). Staining of live cells with monoclonal antibodies had no effect on cAMP levels (Wiemelt et al., 1997). In the absence of agonists, but with PDE inhibitors present, only very low basal levels of cAMP could be detected in any of the cells (Fig. 1A–C). However, addition of calcitonin (150 nM), CRH (150 nM), or VIP (10 nM) for 45 min resulted in a dramatic increase in cAMP immunostaining within individual oligodendroglial cells (Fig. 1D–L, Table I). When A2B5, O4, and O1 antibody were used singly to identify cells, we observed that calcitonin and VIP stimulated cAMP in similar populations of immature cells, consisting of A2B5-positive precursors and O4-positive immature oligodendroglia, but not in O1-positive oligodendrocytes (not shown). CRH was effective somewhat later in development, inducing cAMP content in O4-positive cells with an extensive process network characteristic of mature cells as well as in O1-postive oligodendrocytes (not shown). Each agonist induced a cAMP response in only a minority of the oligodendroglial cells expressing a given developmental marker, suggesting that there is heterogeneity among oligodendroglial cells in their ability to respond to these three agonists. No cAMP elevation was detected in nonoligodendroglial cells in response to calcitonin, CRH, or VIP (Fig. 1D–L, cf. Fig. 2; Table I). Similar results were seen in the presence or absence of PDGF and FGF-2, in the presence or absence of 1%

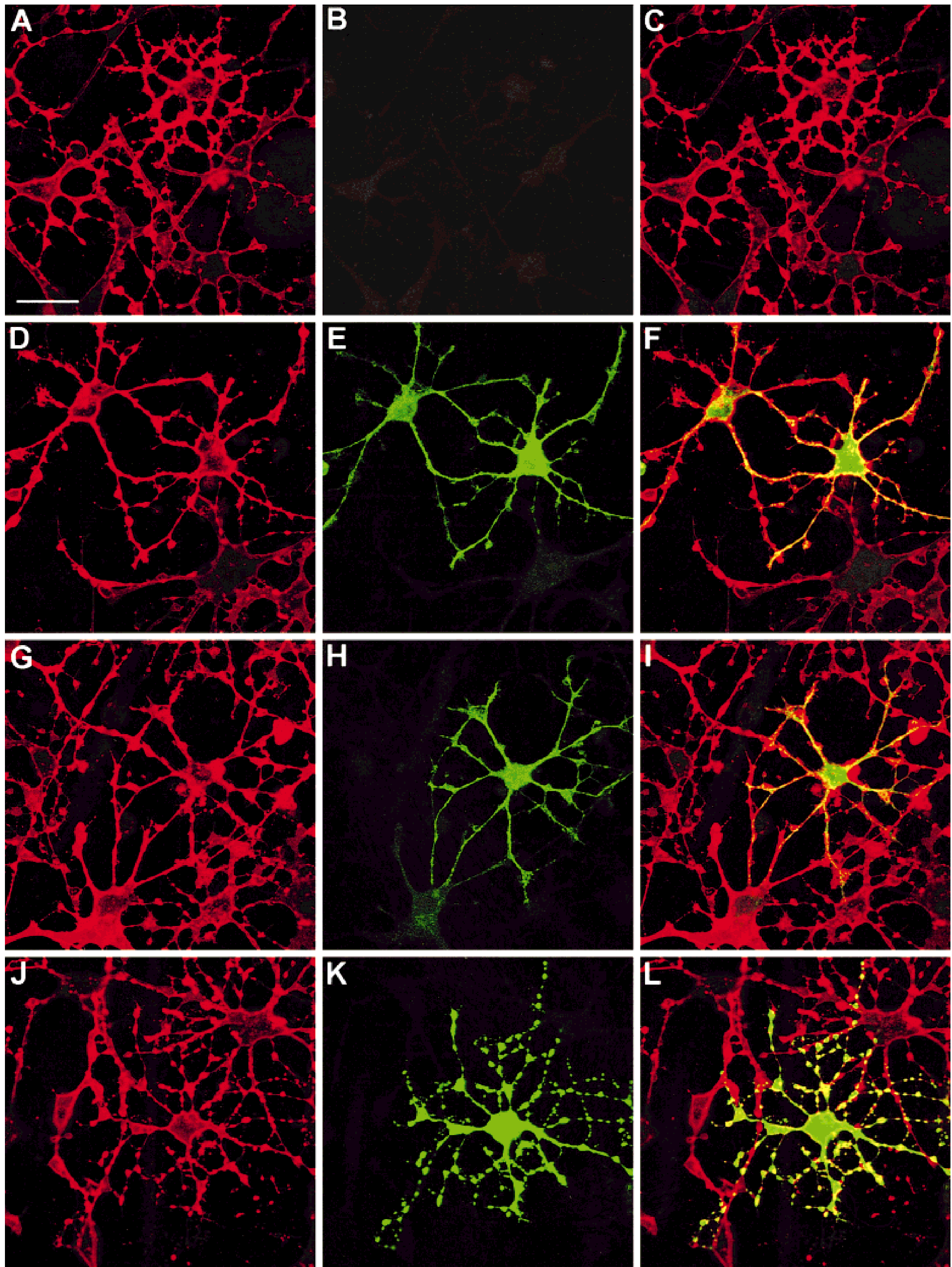

Fig. 1. VIP, calcitonin, and CRH elevate cAMP immunofluorescence in oligodendroglia. Oligodendroglia were grown, treated with PDE inhibitors and cAMP agonists, and stained as described in Materials and Methods and in the footnote to Table I. For each series of panels, cAMP immunofluorescence is shown in green (**B,E,H,K**). Cells were stained with Texas red-conjugated secondary antibody to detect cells labeled with a mixture of A2B5, O4, and O1 antibodies (**A,D,G,J**; overlay shown in **C,F,I,L**). Control cells (A–C) were pretreated with PDE inhibitors, maintained for an additional 45 min in the continued presence of PDE inhibitors but without agonist, fixed, and stained with anti-cAMP antibody. Treatment for 45 min with VIP (D–F), calcitonin (G–I), or CRH (J–L) stimulated cAMP production in oligodendroglia. Confocal sections approximately 0.7 μm thick were taken at the midpoint of the cell. Scale bar = 25 μm.

TABLE I. Cyclic AMP Responses of Oligodendroglial and Nonoligodendroglial Cells to Agonists*

				Response to agonist:	
Agonist/mixture	Concentration	References	Source	OL	Non-OL
Adenosine mix				−	+++
Adenosine	100 μM	1	Sigma/RBI		
8-Cyclopentyl-1,3-dipropylxanthine	3 μM	2	Sigma/RBI		
CGS-21680	3 mM	3	Sigma/RBI		
β-Adrenergic mix				−	+++
(−)Isoproterenol	10 μM	4–6	Sigma/RBI		
Phenoxybenzamine	15 μM	4–6	Sigma/RBI		
Calcitonin	150 nM	7	Sigma/RBI	++	−
Calcitonin gene-related peptide	10 nM	8	Sigma/RBI	−	+
Corticotropin-releasing hormone	150 nM	9	Sigma/RBI	++	−
Dopamine mix				−	+
Sulpiride	1 μM	10,11	Sigma/RBI		
SKF38393	1 μM	10,11	Sigma/RBI		
Epidermal growth factor	90 nM	12	Sigma/RBI	−	+++
Follicle-stimulating hormone	20 U/ml	13	Sigma/RBI	−	++
Glucagon	25 nM	14	Sigma/RBI	−	+
Histamine	1 μM	15	Sigma/RBI	−	+
Prostaglandin (PG) D1	1 μM	16	Sigma/RBI	−	+++
PGE1	1 μM	17	Calbiochem	−	+++
PGE2	1 μM	16	Calbiochem	−	+++
PGF2α	1 μM	16	Sigma/RBI	−	+
PGI2	1 μM	16	Sigma/RBI	−	+++
Serotonin mix				−	+
Serotonin	17.6 μM	18,19	Sigma/RBI		
Fluoxetine	0.7 μM	18,19	Sigma/RBI		
Pindolol	1 μM	18,19	Sigma/RBI		
Substance P	440 nM	20	Sigma/RBI	−	+
Vasoactive intestinal peptide	10 nM	21	Sigma/RBI	++	−

*Summary of the responses of cells in glial cell cultures to putative cAMP agonists. Purified oligodendroglial cell cultures were pretreated for 30 min with 1 mM IBMX and 80 μM rolipram, challenged with various cAMP agonists or agonist mixtures as shown for 45 min in the presence of antibodies to stain cell type-specific surface markers, fixed, and stained with anti-cAMP antibody as described in Materials and Methods. Efficacy of agonists was scored as the number of cells that were immunostained for cAMP and the intensity of the cAMP staining: −, no response; +, 1–3 cells per field responded weakly; ++, 1–3 cells per field responded robustly; +++, 4+ cells per field responded robustly. References: [1]Peakman and Hill (1994); [2]Shryock et al. (1993); [3]Hasko et al. (1996); [4]McMorris (1977); [5]Cohen and Almazan (1994); [6]Guellaen and Hanoune (1979); [7]Byfield et al. (1976); [8]Morris et al. (1984); [9]Rivier et al. (1984); [10]Pollack and Fink (1996); [11]Farooqui (1994); [12]J. Grinspan (personal communication); [13]Grasso and Reichert (1989); [14]Foster (1984); [15]Xia et al. (1996); [16]G. DeVries, (personal communication); [17]McCarthy and de Vellis (1980); [18]Glennon et al. (1987); [19]Ni and Miledi (1997); [20]Marathe et al. (1996); [21]Stryjek-Kaminska et al. (1996).

serum, or after agonist treatment times ranging from 30 to 90 min (data not shown).

Several previous publications have reported that β-adrenergic agonists such as isoproterenol elevate cAMP content in oligodendrocytes (McCarthy and de Vellis, 1980; Vartanian et al., 1988; Cohen and Almazan, 1994; Ghiani et al., 1999). In each of these studies, oligodendrocyte-enriched glial cell cultures were exposed to agonist, and then cells were homogenized and cAMP was determined by biochemical assay. However, in our study, we examined cAMP levels in individually identified oligodendrocytes. Isoproterenol was added to the culture medium either by itself or together with the selective α-adrenoceptor antagonist phenoxybenzamine to block isoproterenol-dependent inhibition of adenylate cyclase. Isoproterenol failed to elevate cAMP immunofluorescence in oligodendroglial cells both in the presence of phenoxybenzamine (Fig. 2A–C) and in its absence (not shown). Essentially identical results were observed in the presence of PDGF and FGF-2 (not shown). However, nonoligodendroglial cells in the culture, which resembled astrocytes or microglia morphologically, responded robustly to these treatments (Fig. 2A–C).

PGE1 has also been reported to elevate cAMP in oligodendroglial cell preparations as determined by biochemical assay (McCarthy and de Vellis, 1980). In our experiments, however, PGE1, PGE2, PGD2, and PGI2 had no detectable effect on cAMP levels in oligodendroglia, but they produced remarkably robust responses in nonoligodendroglial contaminants in these cultures (PGE1 and PGE2 responses are shown in Figure 2D–F and G–I, respectively; others not shown). PGF2α was also tested but showed only weak elevation of cAMP immunofluorescence in astrocyte-like cells (not shown).

The responses of oligodendroglial and nonoligodendroglial cells to agonists are summarized in Table I. Most agonists elevated cAMP in either oligodendroglial or

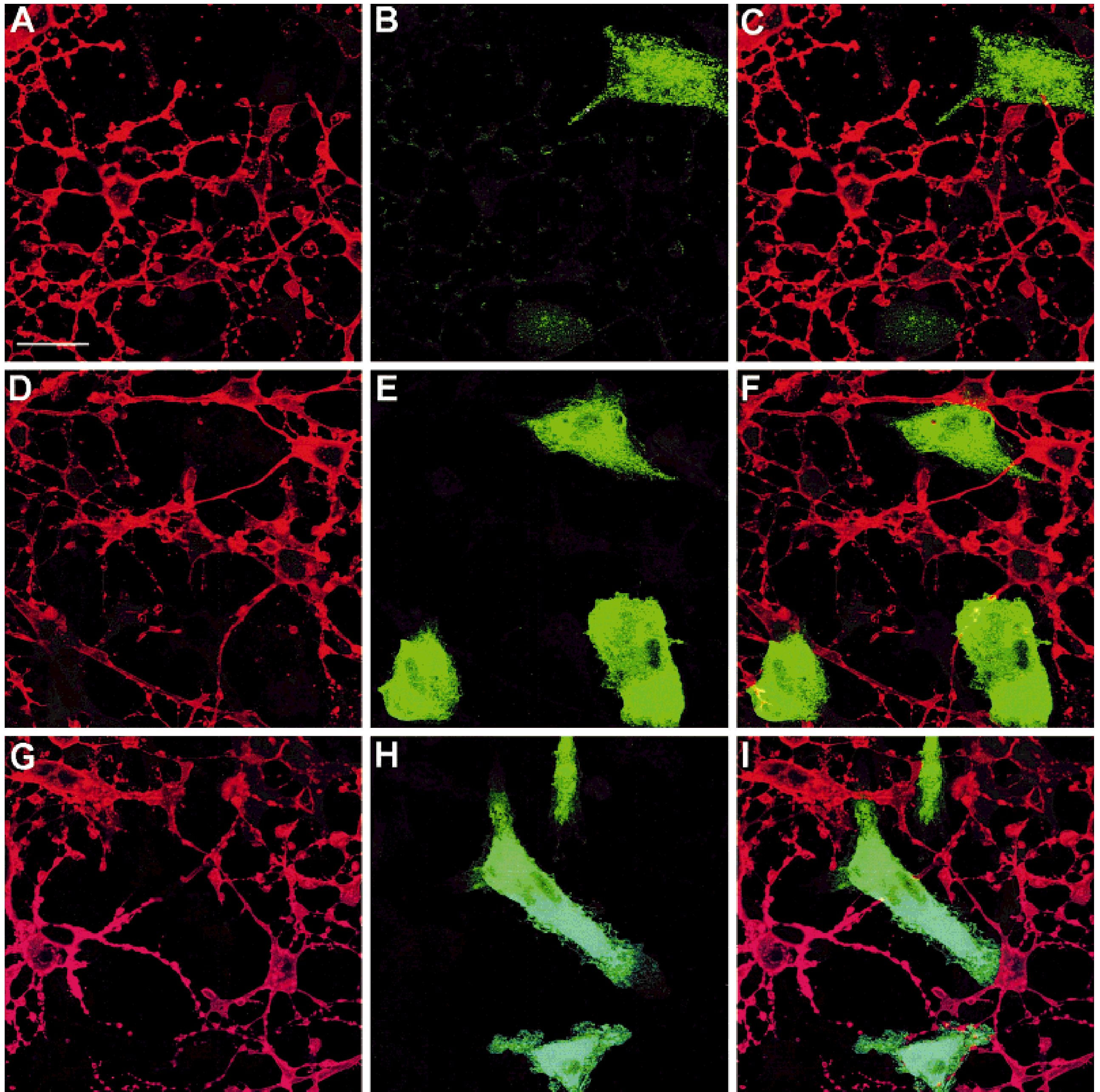

Fig. 2. Isoproterenol and prostanoids elevate cAMP in nonoligodendroglial cells but not in oligodendroglial cells. Cultures were grown and treated as described in the legend to Figure 1. Cells were stained with a mixture of A2B5, O4, and O1 antibodies (Texas red; **A,D,G**) to detect oligodendroglia. cAMP immunofluorescence is shown in **B,E,H**; overlay is shown in **C,F,I**. Isoproterenol with phenoxybenzamine (A–C) elicited cAMP elevation in nonoligodendroglial cells. PGE1 (D–F) and PGE2 (G–I) markedly elevated cAMP in nonoligodendroglial cells but did not increase cAMP immunofluorescence detectably in oligodendroglial cells. Confocal images were taken as described in the legend to Figure 1. Scale bar = 25 μm.

nonoligodendroglial cells, which thus served as convenient internal positive controls for the efficacy of the agonist treatments and the success of the immunostaining procedure. Significantly, agonists that elicited responses from oligodendroglia showed no detectable activity toward nonoligodendroglial cells, whereas agents producing cAMP elevations in nonoligodendroglial cells had no detectable effect in oligodendroglia.

DISCUSSION

By directly visualizing cAMP in cells that were individually identified as oligodendroglia by immunochemi-

cal criteria, we were able to identify several agonists that elevate cAMP in oligodendroglia. Furthermore, we were able to observe that the responsiveness of oligodendroglial cells to these agonists varies with the developmental stage of the cell and also varies from cell to cell among cells of the same apparent developmental age. Finally, our analysis revealed that several agonists previously thought to stimulate cAMP elevation in oligodendroglia actually fail to do so.

The ability of calcitonin, CRH, and VIP to elevate cAMP in oligodendroglia was intriguing; their effects have never been examined in oligodendroglia. VIP and melanocortins have been shown to elevate cAMP in Schwann cells (Yasuda et al., 1988; Dyer et al., 1995). VIP at 10^{-10} M stimulates the release of neurotrophic factors and maximally stimulates proliferation in astrocytes (Brenneman et al., 1990; Gozes et al., 1991; Martin et al., 1992), although calcium signaling, rather than cAMP, may transduce these signals (Fatatis et al., 1994). CRH receptors are widely distributed in the brain, where they are positively coupled to cAMP production (DeSouza et al., 1985). Calcitonin gene-related peptide (CGRP) has been reported to act in Schwann cells (Cheng et al., 1995), although in our study calcitonin and not CGRP stimulated cAMP immunofluorescence in oligodendroglia. It is important to determine whether these agonists affect developmental processes in oligodendrocytes in vitro and in vivo.

The populations of oligodendroglia that were activated by these agonists were restricted to earlier developmental stages. VIP acted in the earliest population of cells studied, elevating cAMP in A2B5-postive and early O4-positive precursors. Consistently with this observation, Vartanian et al. (1988) noted that down-regulation of VIP receptors in oligodendroglia occurred soon after cells adhered to substrate, indicating that they might be expressed in early, less well-differentiated precursors. Whereas cells beyond the intermediate/late precursor stage are considered "mature" oligodendrocytes, cells at this earlier stage (O4-positive, O1-negative) are not fully committed and can dedifferentiate to proliferative, mobile cells (McKinnon et al., 1993). Precise regulation of events leading to this transition is undoubtedly important for the precise control of development. Only a minority of cells within the A2B5-positive and the O4-positive populations was responsive, indicating that there is heterogeneity even among cells expressing the same developmental marker.

Whereas previous biochemical studies have reported the elevation of cAMP in oligodendrocyte-enriched glial cell cultures in response to β-adrenergic agonists and PGE1, we were unable to observe elevated cAMP immunofluorescence in individually identified oligodendroglial cells in response to these agonists. Consistently with our findings, autoradiographic quantitation of β-adrenergic receptors in primary cultures of cerebral cortical cells demonstrated abundant expression of β-adrenoreceptors in astroglia, whereas expression in oligodendrocytes was undetectable (Burgess et al., 1985). "Pure" oligodendroglial cell cultures, as those used in previous studies of cAMP are reported to be, are actually 90–98% oligodendroglial cells (McCarthy and de Vellis, 1980; Cohen and Almazan, 1994). However, a 2–10% contamination by nonoligodendroglial cells could readily account for a modest increase in cAMP level in the homogenate. β-Adrenergic stimulation of clonal glial cell lines has been reported to elevate cAMP content by 250-fold or even more (McMorris, 1983b). In contrast, cAMP elevation in homogenates of oligodendroglial cell cultures treated with β-adrenergic or other agonists, as measured biochemically, is reported to be a modest two- to fivefold (McCarthy and de Vellis, 1980; Cohen and Almazan, 1994) to as much as 40-fold (Ghiani et al., 1999). A response of this magnitude could be accounted for by routine astrocyte contamination of the cultures. Ghiani et al. (1999) reported that treatment of oligodendrocyte-enriched glial cell cultures with isoproterenol or norepinephrine inhibited the proliferation and promoted the maturation of oligodendrocytes and that these responses could be mimicked by cAMP analogs. Because the cultures contain nonoligodendroglial cells such as astrocytes, which are known to produce a number of factors that regulate oligodendrocyte growth and development (Ballotti et al., 1987; Richardson et al., 1988; Barres et al., 1994), β-adrenergic stimulation of these cultures may affect oligodendroglia indirectly by eliciting a cAMP-mediated response from these contaminating cells.

For PGE1, McCarthy and de Vellis (1980) reported that populations of relatively pure oligodendroglia responded much more robustly than did enriched astrocyte cultures. In this case, the cAMP response of the oligodendroglial cell cultures cannot be readily attributed to contaminating astrocytes but may instead be accounted for by the presence of microglia, which respond robustly to prostanoids and are routinely present in "purified" oligodendroglial cell cultures (Behar et al., 1988; Caggiano and Kraig, 1999).

Because previous studies have shown that growth factors can regulate receptor expression, we tested oligodendroglia grown with and without the growth factors PDGF and FGF-2, including the same culture conditions and medium formulations used in previous studies of cAMP responses in oligodendrocytes (Cohen and Almazan, 1994; Ghiani et al., 1999). With these conditions our results were essentially the same as those with our standard conditions: Isoproterenol and PGE1 induced cAMP levels robustly in nonoligodendroglial cells but not in oligodendroglial cells.

In summary, we have identified, for the first time, receptor agonists that activate cAMP synthesis within individually identified oligodendroglial cells. It is now important to determine whether these agonists are capable of regulating developmental processes in oligodendroglia in a manner similar to that reported for forskolin, cholera

toxin, or cAMP analogues such as dibutyryl cAMP and 8-bromo-cAMP.

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