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Acetylcholine as a mitogen: muscarinic receptor-mediated proliferation of rat astrocytes and human astrocytoma cells

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Abstract

The mitogenic effect of muscarinic receptor agonists in glial cells has been characterized in rat cortical astrocytes and human 132 1N1 astrocytoma cells. The muscarinic receptor agonist carbachol caused a dose- and time-dependent increase in proliferation, as measured by [^{3}H]thymidine incorporation. The mitogenic effect was mimicked by several muscarinic, but not nicotinic receptor agonists, and was blocked by muscarinic receptor antagonists. Reverse transcription-polymerase chain reaction (RT-PCR) experiments indicated the presence of m2, m3 and to a lesser degree, m5 muscarinic receptor mRNA in both astrocytes and astrocytoma cells. Proliferation experiments with subtype-specific muscarinic receptor antagonists suggest that carbachol-induced proliferation is due to activation of muscarinic M_3 receptors. The phorbol ester 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) also stimulated glial cell proliferation. Down-regulation of protein kinase C, or the protein kinase C antagonist 1,5-(isoquinolynsulfanyl)-2-methylpiperazine dihydrochloride (H7) blocked proliferation induced by either TPA or carbachol. Of other neurotransmitters tested, histamine caused glial cell proliferation, norepinephrine and γ-aminobutyric acid were ineffective, while serotonin and glutamate inhibited basal or serum-stimulated proliferation.

Keywords: Muscarinic receptor; Cell proliferation; Astrocyte; Astrocytoma

1. Introduction

Receptors for a variety of neurotransmitters have been identified on astrocytes in the mammalian central nervous system, where they can regulate several second messenger pathways (Hosli and Hosli, 1993). Although the presence of acetylcholine muscarinic receptors in glial cells had been questioned by earlier studies (Gonzales et al., 1985), a large body of evidence has since accumulated which indicates that muscarinic receptors are present in astrocytes. In these cells, muscarinic receptor agonists have been shown to cause hydrolysis of membrane phosphoinositides and mobilization of intracellular Ca^{2+} (Enkvist et al., 1989), to activate phospholipase D (Gustavsson et al., 1993) and phospholipase A_2 (De George et al., 1986) activities, to inhibit adenylate cyclase activity (Murphy et al., 1986), and to induce immediate-early-genes, such as *c-fos* and *c-jun* (Trejo and Brown, 1991).

Less is known on the consequences that activation of muscarinic receptors may have with regard to astrocyte function. Muscarinic receptor agonists failed to alter glycogen content (Cambray-Deakin et al., 1988) and hyposmotic swelling (Bender et al., 1993); on the other hand, they have been shown to induce the synthesis and release of nerve growth factor (Furukawa et al., 1987). An important observation was made a few years ago by Ashkenazi et al. (1989), who showed that activation of muscarinic receptors coupled to phosphoinositide metabolism (m1, m3, m5), but not of those preferentially coupled to inhibition of adenylate cyclase (m2, m4), causes proliferation of rat cortical astrocytes. The mitogenic effect of acetylcholine in astrocytes may be relevant in terms of brain development, as proliferation of these cells is a major event during the brain growth spurt (Bayer et al., 1993). Furthermore, as phosphoinositide metabolism stimulated by muscarinic receptor agonists is enhanced during the brain growth spurt (the first two postnatal weeks in the rat; Balduini et al., 1987), this second messenger pathway may be relevant for the mitogenic action of acetylcholine.

These considerations prompted us to reexamine and further characterize the mitogenic effect of muscarinic

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receptor agonists in rat cortical astrocytes as well as in human astrocytoma cells, which also possess functional muscarinic receptors (Masters et al., 1984). Additionally, since the subtypes of muscarinic receptors have not been precisely identified in these cells (Murphy et al., 1986; Stephan and Sastry, 1992), we conducted experiments to identify the subtype(s) of muscarinic receptor mRNA present.

2. Materials and methods

2.1. Materials

Pregnant Sprague-Dawley rats were supplied by B&K Universal (Kent, WA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum and trypsin were purchased from GIBCO (Grand Island, NY, USA). Glial fibrillary acidic protein (GFAP), neuron-specific enolase and fibronectin antibodies were purchased from Accurate Chemical (Westbury, NY, USA). [methyl-^{3}H]Thymidine (6.7 Ci/mmol) was from New England Nuclear (Boston, MA, USA). Methoctramine, 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide), HHSiD (hexahydro-siladifenidol hydrochloride, *p*-fluoro analogue) and H7 (1-5(-isoquinolinylsulfonyl)2-methylpiperazine dihydrochloride) were obtained from Research Biochemicals (Natick, MA, USA). Mecamylamine HCl was a gift from Merck (Rahway, NJ, USA). Agarose was from FMC (Rockland, ME, USA). DNAse I enzyme, oligo d(T)-(12–18) primer, 100 mM dNTP solutions, RNAguard ribonuclease inhibitor, AMV Reverse Transcriptase were purchased from Pharmacia-LKB (Piscataway, NJ, USA). Taq DNA polymerase, T4 polynucleotide kinase and 123 bp DNA ladder size

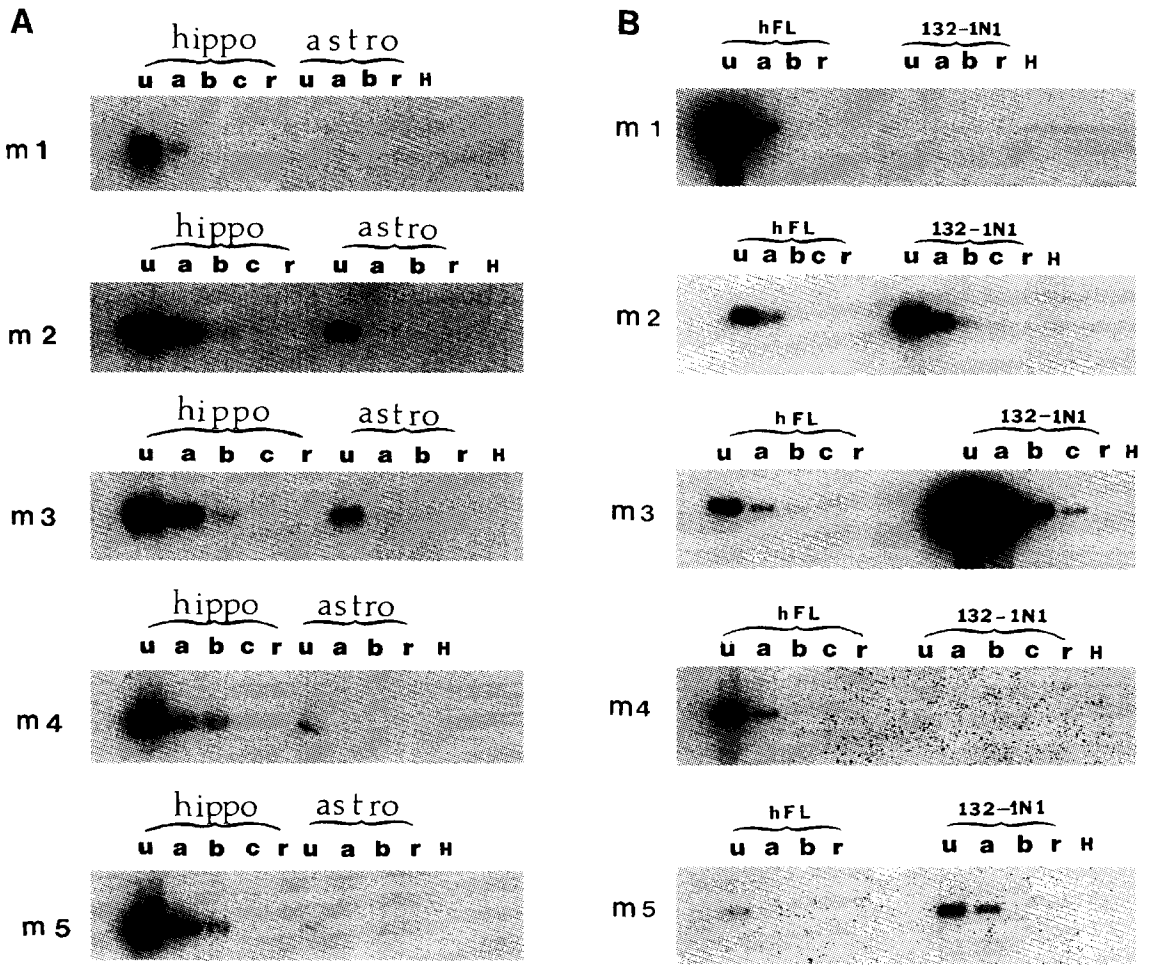

Fig. 1. (A) Expression of muscarinic receptor subtype mRNAs in rat astrocytes as detected by RT-PCR followed by Southern blot and hybridization with internal probe. A rat hippocampus tissue sample (hippo) was used as positive control for the presence of all five subtypes of muscarinic receptor and to compare the relative intensities of bands with the cortical astrocytes sample (astro). u: 500 ng of undiluted cDNA; a,b,c: 10-fold, 100-fold, 1000-fold, respectively, cDNA dilutions; r: DNAseI-treated RNA that had not been reverse transcribed was used as PCR template. (B) Analysis of muscarinic receptor subtypes expression in human 132 1N1 astrocytoma cells. The positive control for the presence of all five subtypes of muscarinic receptors was a human frontal lobe sample (hFL). Samples were subjected to RT-PCR and Southern blot as described in Methods. u: 500 ng of undiluted cDNA; a,b,c: 10-fold, 100-fold, 1000-fold cDNA dilutions; r: DNAseI-treated RNA that had not been reverse transcribed was used as PCR template. H: H_2O.

markers were from BRL (Gaithersburg, MD, USA). GeneScreenPlus nylon membranes and [γ-^{32}P]dATP were from NEN-DuPont (Wilmington, DE, USA). Films used for autoradiography were Kodak XAR. Oligomers used for polymerase chain reaction (PCR) were synthesized by the Howard Hughes Medical Institutes at the University of Washington with an Applied Biosystems 380A synthesizer. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Methods

2.2.1. Primary astrocyte cultures

Primary cultures of astrocytes were prepared from cerebral cortex of 21-day-old rat fetuses by a modification of the method of McCarthy and de Vellis (1980). Briefly, the tissue, free of meninges, was dissected to 1–2 mm^3 sections in Hanks' balanced salt solution (Ca^{2+},Mg^{2+}-free) and incubated for 10 min in 0.2% trypsin at 37°C. Trypsinization was terminated by adding DMEM supplemented with 10% fetal bovine serum, and the cells were sedimented at 1000 rpm for 10 min, resuspended in DMEM/10% fetal bovine serum and vortexed at maximum speed for 1 min. After three washes at 1000 rpm for 10 min, the cells were resuspended in the same medium, filtered through a nylon mesh of 100 μm pore size, plated in poly-D-lysine coated 75 cm^2 flasks at a concentration of 1.6×10^5 cells/cm^2, and incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air in DMEM supplemented with 10% fetal bovine serum, 1 U/ml penicillin and 1 μg/ml streptomycin. The culture medium was renewed every 4 or 5 days. After 9 days, oligodendrocytes and microglia were removed from cultures by vigorously shaking the flasks overnight. Neuronal cells were eliminated by feeding twice with iced-cold PBS and by cell detaching with 0.25% trypsin. Astrocytes were then replated in poly-D-lysine-precoated 24 well culture plates at a concentration of 10^6 cells/well. This rigorous passing at high density limited microglial survival (Mudd and Raizada, 1990). After 1 day, the cells were shifted in the same medium without serum supplemented with 0.1% bovine serum albumin (essential fatty acid free) for 48 h, and cells were then used for proliferation assay. The culture's cellular homogeneity was determinated by indi-

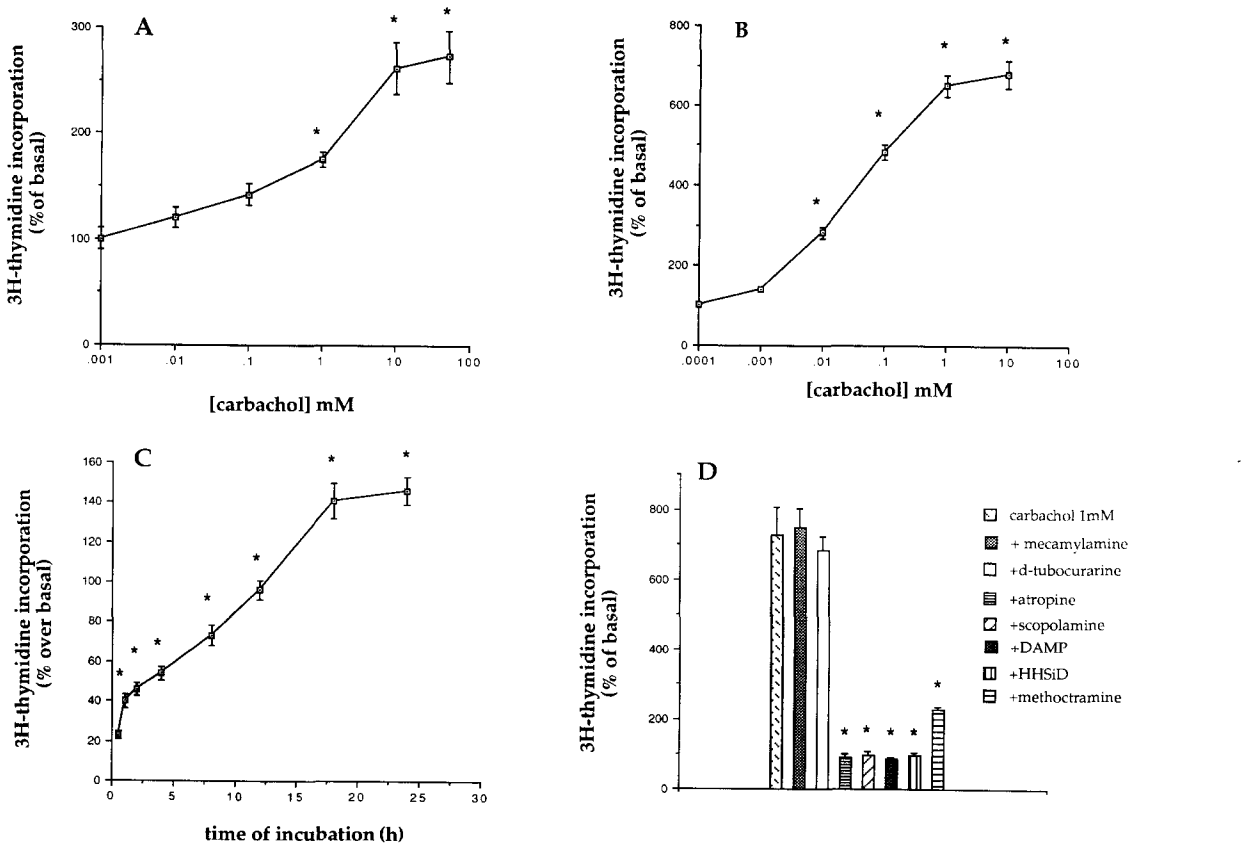

Fig. 2. (A,B) Concentration-response effect of carbachol on [^{3}H]thymidine incorporation in astrocytes (A) and 132 1N1 astrocytoma cells (B). Results are expressed as percentages of basal. (C) Time-course of carbachol (10 mM)-induced stimulation of [^{3}H]thymidine incorporation in rat cortical astrocytes. Results are expressed as percentages proliferation over basal. (D) Effect of muscarinic and nicotinic receptor antagonists on carbachol (1 mM)-induced [^{3}H]thymidine incorporation in 132 1N1 human astrocytoma cells. Concentration of antagonists is 10 μM. Results are expressed as percentages of basal. Basal incorporation of [^{3}H]thymidine was (cpm/well): 10870 ± 1076 and 6185 ± 310 for astrocytes and astrocytoma cells, respectively. All data points represent the means (± S.E.M.) of at least six observations. * $P < 0.05$.

rect immunofluorescence labeling. Staining for fibroblasts (fibronectin) was never detected in our preparations. Staining for neuron-specific enolase was rarely observed, and only up to 14 days in culture. Routine cell preparations stained > 95% GFAP-positive.

In experiments where muscarinic receptor mRNAs were measured, primary culture of cortical astrocytes were prepared as described above, however, the cells were kept in culture for 28 days in order to minimize any possible contamination by other cell types. The astrocytes were shaken overnight and trypsinized 3 times, then reseeded in poly-D-lysine 75 cm^2 flasks at the concentration of 7×10^6 cells/flask. These treatments select for a relatively pure population of type 1 astrocytes (Bal et al., 1994). These cultures stained 99% GFAP positive.

2.2.2. 132 1N1 human astrocytoma cells

The human astrocytoma cell line 132 1N1 (kindly donated by Dr Joan H. Brown, San Diego, CA, USA) was maintained in DMEM, low glucose, supplemented with 5% fetal bovine serum, 1 U/ml penicillin G and 1 mg/ml streptomycin in 75 cm^2 flasks under a humidified atmosphere of 5% CO_2-95% air at 37°C. Cells were subcultured every 7 days and the growth medium was changed every 3 or 4 days. For the [^{3}H]thymidine experiments, cells were seeded in 24 well plates and shifted in the same medium without serum supplemented with 0.1% bovine serum albumin.

2.2.3. Proliferation assay

Incorporation of [methyl-^{3}H]thymidine into cell DNA was measured as described by Freshney et al. (1980), with slight modifications. After 48 h in serum-free medium, agonists were added for 24 h. One μCi/well [methyl-^{3}H]thymidine was included for the last 6 h of the incubation at 37°C under an atmosphere of 5% CO_2-95% air. Cells were washed twice with cold PBS and fixed in methanol. Unincorporated precursor was removed by two washes with ice-cold 10% trichloroacetic acid and one wash of ice-cold 0.5% trichloroacetic acid. The monolayer was dissolved in 500 μl of 1 N NaOH and 250 μl were transferred to scintillation fluid and counted on a Beckmann LS 5000 CE scintillation counter. In the time-course study, cells were incubated for 0.5–24 h with carbachol and then returned to serum-free medium until the addition of [^{3}H]thymidine.

2.2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from rat astrocytes and human 132 1N1 astrocytoma cells by the AGTPC method (Chomcyznski and Sacchi, 1987) and treated with DNAseI to remove possible traces of contaminating genomic DNA. Five μg of DNAseI-treated RNA for each sample were used to make cDNA in a reverse-transcription reaction. The conditions of DNAse I treatment and cDNA synthesis were as previously described (Costa et al., 1994). Undiluted cDNAs, as well as serial dilutions of them, were used in PCR reactions using primers specific for each subtype of rat or human muscarinic receptor. The PCR products were subjected to electrophoresis and Southern blot and hybridized with a radiolabeled probe with a sequence internal to the PCR products. Primers and conditions of PCR used for the analysis of expression of rat m2, m3, m4 and m5 muscarinic receptor subtypes were as previously described (Costa et al., 1994). Primers for rat muscarinic m1 receptors were as follows: a forward primer corresponding to bases 860–878: 5′-AGCTCAGAGAGGTCACAG-3′ (Eva et al., 1992), a reverse primer complementary to bases 1130–1150: 5′-TCGGTCTCGGCCTTTCTTGGT-3′ (McKinney and Robbins, 1992). PCR annealing temperature was 51.5°C. The m1 PCR product hybridization probe was designed based on the GenBank data base, complementary to bases 976–1000: 5′CTCCATGGAGCCTTCATCCTCCTC-3′. Hybridization temperature was 51°C.

The Forward Primer (FP) and Reverse Primer (RP) for the analysis of expression of the five subtypes of human muscarinic receptors in 132 1N1 astrocytoma cells were as

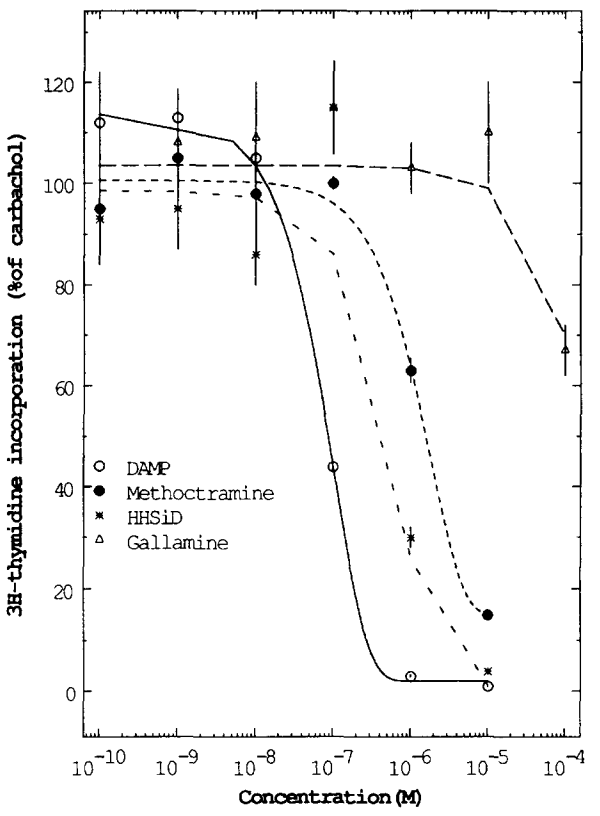

Fig. 3. Concentration-response inhibition of carbachol (1 mM)-stimulated proliferation of 132 1N1 astrocytoma cells by the muscarinic M_2 receptor antagonists methoctramine and gallamine, and the muscarinic M_3 receptor antagonists 4-DAMP and HHSiD. Values of IC_{50} were: 90 ± 2 nM (4-DAMP); 130 ± 10 nM (HHSiD); 2.8 ± 0.7 μM (methoctramine). Each point represents the mean (± S.E.M.) of three separate experiments.

published by Baumgartner et al. (1993): hm1 FP: bases 826–847; hm1 RP: bases 1378–1399; hm2 FP: bases 443–464; hm2 RP: bases 891–912; hm3 FP: bases 531–552; hm3 RP: bases 1365–1377; hm4 FP: bases 76–98; hm4 RP: bases 703–724; hm5 FP: bases 654–675; hm5 RP: bases 1433–1454. The hybridization probes for all five human subtypes were designed based on the coding sequence obtained from the GenBank data base, as detailed in Costa et al. (1995). As a control for the efficiency of cDNA preparation, PCR was performed also for the human cytoplasmic β-actin. The sequences of the mouse/rat/human β-actin forward and reverse primers were as follows: β-actin forward primer: bases 2168–2193; β-actin reverse primer: bases 3053–3078 (Krapf and Solioz, 1991).

2.3. Statistical analysis

In [methyl-^{3}H]thymidine incorporation assays, triplicate culture wells were used, and each experiment was performed at least twice. All statistical tests were carried out using the STATVIEW 512 program in a Macintosh personal computer. One way analysis of variance (ANOVA) followed by the Fisher's Least Significance Difference test were used to determine significant ($P < 0.05$) differences over controls.

3. Results

In order to determine the subtype(s) of muscarinic receptors expressed in rat cortical astrocytes and 132 1N1 human astrocytoma cells, the presence of the corresponding mRNAs was analyzed by RT-PCR. Fig. 1A shows the results of Southern blotting and hybridization with the five muscarinic receptor subtype-specific probes of the PCR products for rat astrocytes. A rat hippocampus tissue sample was used as positive control. The astrocyte culture primarily expressed muscarinic m2, m3 receptor mRNA; traces of m5 receptor mRNA could be detected, while the m1 and m4 receptor subtypes were undetectable. Fig. 1B shows the analysis of PCR products obtained for the muscarinic receptor subtypes in 132 1N1 astrocytoma cells. A human brain frontal lobe tissue sample was used as positive control. Like rat astrocytes, human astrocytoma cells expressed the muscarinic m2, m3 and m5 receptor subtype mRNAs.

Carbachol caused a dose-dependent increase of [^{3}H]thymidine incorporation in both rat cortical astrocytes and human astrocytoma cells (Fig. 2A,B) with EC_{50}s of 520 ± 82 μM and 40 ± 6 μM, respectively ($n = 6$). In these experiments cells were maintained in serum-free medium for 48 h before the addition of carbachol for 24 h.

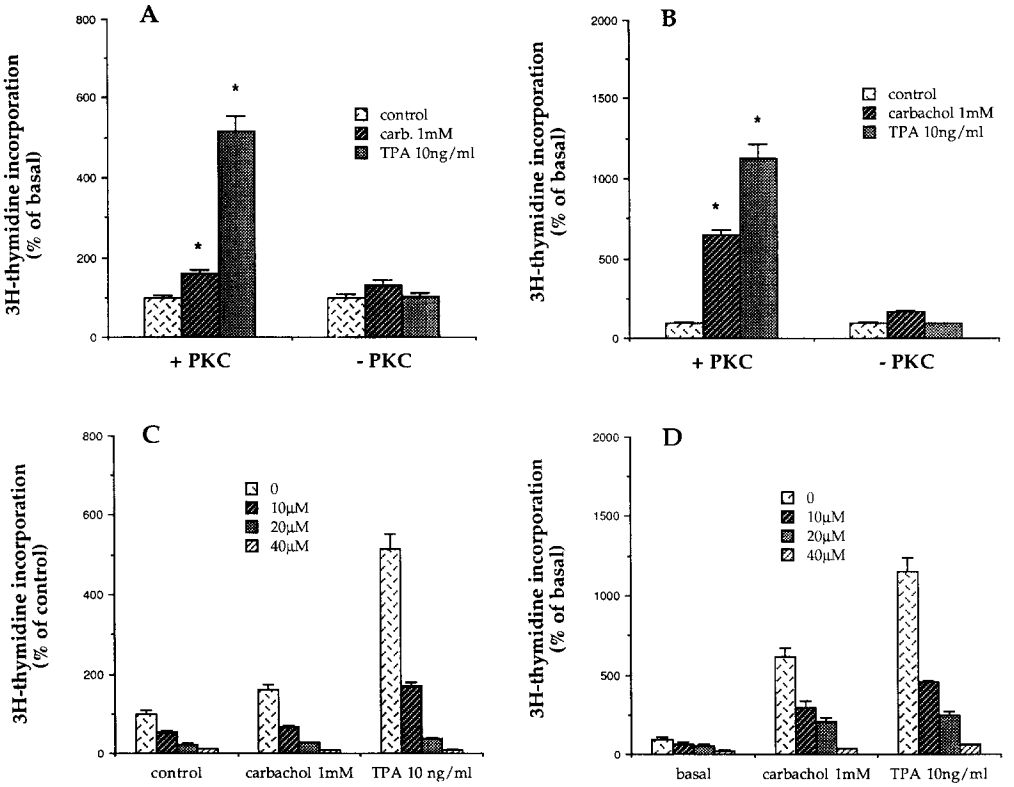

Fig. 4. Effect of protein kinase C depletion (by 300 nM TPA for 24 h) on the induction of [^{3}H]thymidine incorporation by carbachol and TPA in astrocytes (A) and 132 1N1 astrocytoma cells (B). Inhibition by H7 of the stimulation of [^{3}H]thymidine incorporation by carbachol and TPA in astrocytes (C) and 132 1N1 astrocytoma cells (D). Results are expressed as percentages of basal. Basal incorporation of [^{3}H]thymidine was (cpm/well): 10752 ± 720 and 5863 ± 800 in astrocytes and astrocytoma cells, respectively. In cells treated for 24 h with TPA, basal [^{3}H]thymidine incorporation increased by 3-fold. Each bar represents the mean ($\pm$S.E.M.) of at least six observations. * $P < 0.05$.

A time-course study (30 min to 24 h) was conducted in astrocytes (Fig. 2C). Carbachol caused a statistically significant increase in [^{3}H]thymidine incorporation (30% over basal) after 1 h incubation, and proliferation reached a plateau after 18 h incubation. In addition to carbachol, other muscarinic receptor agonists (methacholine, acetylcholine) were also capable of causing proliferation of rat astrocytes and human astrocytoma cells, and their effect was comparable to that of carbachol (not shown). The mitogenic effect of muscarinic receptor agonists was blocked by a number of acetylcholine muscarinic receptor antagonists (atropine, scopolamine, 4-DAMP, HHSiD and methoctramine). Results from experiments with carbachol carried out in astrocytoma cells are shown in Fig. 2D. Similar results were also obtained when muscarinic receptor antagonists were tested against other muscarinic receptor agonists (data not shown). The nicotinic receptor antagonists mecamylamine and *d*-tubocurarine had no effect on carbachol-induced proliferation (Fig. 1D). Furthermore, the nicotinic receptor agonists 1,1-dimethyl-4-phenylpiperazinium (DMPP) and cytisine (both at 100 μM) had no mitogenic effect in astrocytoma cells (100 $\pm$ 8% and 146 $\pm$ 14% of basal, respectively; $n = 3$; $P > 0.05$). None of the muscarinic or nicotinic receptor antagonists had any effect when present alone. Since m2 and m3 muscarinic receptors were found to be expressed in both rat cortical astrocytes and human astrocytoma cells, additional experiments were conducted with the m2 receptor antagonists methoctramine and gallamine and the m3 receptor antagonists 4-DAMP and HHSiD. Fig. 3 shows that both m3 receptor antagonists were more potent than the m2 receptor antagonists in inhibiting carbachol-induced proliferation of astrocytoma cells.

Phorbol esters had been previously shown to cause proliferation of rat astrocytes (Murphy et al., 1987). We confirmed that 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) causes a dose-dependent proliferation of rat cortical astrocytes and human astrocytoma cells. At a concentration of 10 ng/ml, TPA increased proliferation of astrocytes and astrocytoma cells by 515 $\pm$ 35% and 1379 $\pm$ 198% of basal, respectively ($n = 6$; $P < 0.01$). To determine whether activation of protein kinase C was involved in the mitogenic effect of carbachol, stimulation of [^{3}H]thymidine incorporation by carbachol and TPA was measured in protein kinase C-depleted cells. This was accomplished by pre-incubation of cells with 300 nM TPA for 24 h before washing and the addition of agonists, as described by Tranque et al. (1992). Under these conditions, basal proliferation of both cell types was increased by 3-fold over normal values (not shown) in agreement with previous observations (Tranque et al., 1992). Carbachol- and TPA-induced proliferation was almost totally blocked in cells where protein kinase C had been down-regulated (Fig. 4A,B). The effect of the protein kinase C inhibitor H7 was also tested. When H7 was present alone, for 24 h, at concentrations of 10, 20 or 40 μM, it caused a dose-dependent decrease in [^{3}H]thymidine incorporation (Fig. 4C,D). H7 also caused a strong, dose-dependent inhibition of proliferation induced by carbachol or TPA in rat cortical astrocytes and human astrocytoma cells (Fig. 4C,D).

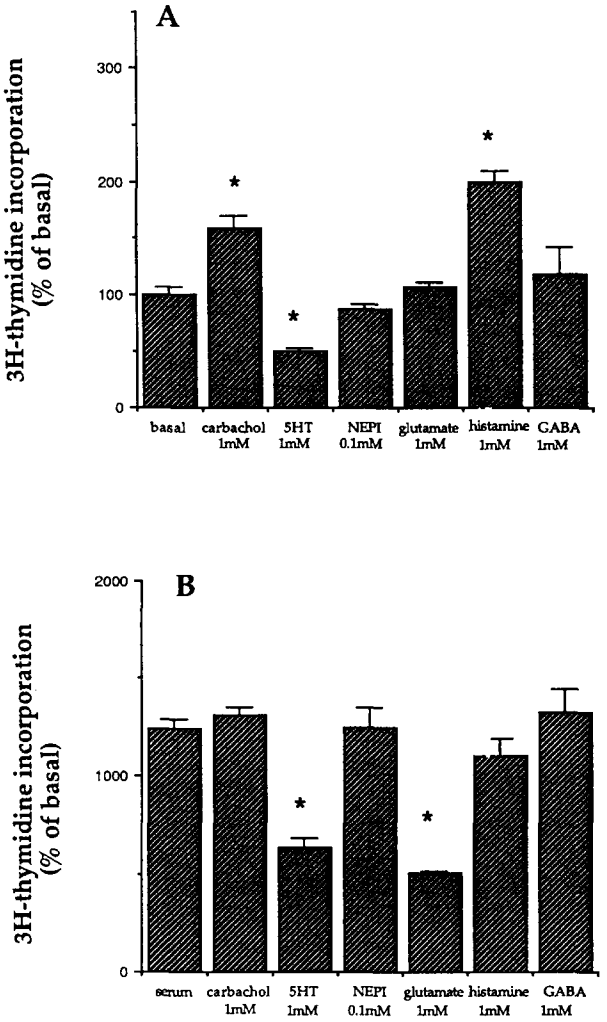

Fig. 5. Effect of neurotransmitters on [^{3}H]thymidine incorporation in rat cortical astrocytes under basal conditions (A) or in the presence of 10% serum (B). Results are expressed as percentages of basal. Each bar represents the mean ($\pm$S.E.M.) of at least six observations. (5HT = 5-hydroxytryptamine; GABA = γ-aminobutyric acid; NEPI = norepinephrine) * $P < 0.05$.

A number of other neurotransmitters (serotonin, norepinephrine, γ-aminobutyric acid (GABA), histamine and glutamate) were also tested for their ability to affect astrocyte proliferation. As shown in Fig. 5A, histamine was a mitogen in rat cortical astrocytes, and has also been shown to cause proliferation of human astrocytoma cells (data not shown). Glutamate, norepinephrine and GABA were ineffective, while serotonin caused a significant decrease in proliferation. Incubation of cells with 10% serum for 24 h caused a 12-fold increase in astrocyte proliferation (Fig. 5B). Under these conditions, carbachol and histamine were ineffective in further stimulating the proliferation. On

the other hand, serotonin, as well as glutamate, caused a profound inhibition of serum-induced proliferation (Fig. 5B). Neither compound caused cytotoxicity, as indicated by the lack of LDH release (control, 12 ± 0.3 U/l; serotonin 14 ± 1.5 U/l; glutamate 14 ± 1.2 U/l; Triton X-100 1%, 102 ± 9; $n = 3$).

4. Discussion

A number of studies have shown that astrocytes and astrocytoma cells possess functional acetylcholine muscarinic receptors (Enkvist et al., 1989; Masters et al., 1984; Stephan and Sastry, 1992). However, the role that such receptors may have in the regulation of glial cell physiology is still not well understood. The observation that muscarinic receptor agonists could stimulate astrocyte proliferation (Ashkenazi et al., 1989; Zohar and Salomon, 1992) prompted us to further investigate their mitogenic effect in rat cortical astrocytes as well as in human 132 1N1 astrocytoma cells. One of the novel findings of the present study is the identification of the subtype of muscarinic receptors expressed in these cells. Utilizing RT-PCR we found that rat cortical astrocytes express mRNA for the m2 and m3 subtypes, which should be responsible for the reported ability of muscarinic receptor agonists to inhibit adenylate cyclase and stimulate phosphoinositide metabolism, respectively, in these cells. Indeed, while earlier pharmacological studies had suggested the presence of muscarinic m1 receptors in astrocytes (Murphy et al., 1986), more recent ones have ascribed the stimulation of phosphoinositide metabolism to the m3 subtype (Kondou et al., 1994). Trace levels of m5 mRNA were also detected in rat cortical astrocytes, however, their functional significance at such low level is questionable. As muscarinic m5 receptors are expressed at low, similar levels in most brain areas (Wei et al., 1994), and represent the major subtype in microglia (Ferrari-DiLeo and Flynn, 1995), the very low levels of m5 mRNA that we detected in astrocytes may be due to microglial contamination. In astrocytoma cells, muscarinic m2, m3 and m5 receptor mRNA was detected by RT-PCR. The presence of muscarinic m2 and m3 receptors had been inferred by previous pharmacological studies (Stephan and Sastry, 1992) and muscarinic m3 receptors were recently identified in astrocytoma cells with a specific antibody (Wall et al., 1991).

Carbachol caused a concentration-dependent increase in the proliferation of rat astrocytes and human astrocytoma cells. The effect was more pronounced in the latter (6–9-fold over basal) than in astrocytes (2.5–3-fold). This difference may be ascribed to a higher density of muscarinic receptors, to a higher expression of one or more intracellular pathways involved in proliferation, or to other cellular factors intrinsic to the transformed cell line. As among muscarinic receptor subtypes those coupled to phosphoinositide metabolism were reported to cause proliferation (Ashkenazi et al., 1989), the presence of both m3 and m5 receptors in astrocytoma cells may also be responsible for the observed higher proliferative response. The mitogenic effect of carbachol was clearly due to the activation of muscarinic receptors, since it was antagonized by several muscarinic receptor antagonists, but not by nicotinic receptor antagonists. Furthermore, other muscarinic receptor agonists were also mitogenic, while nicotinic receptor agonists were ineffective. Experiments with muscarinic M_2 and M_3 receptor antagonists suggested that carbachol-induced proliferation is primarily due to activation of muscarinic m3 receptors, though a contribution of muscarinic m2 receptor cannot be rule out. A time-course study indicated that incubation with carbachol for at least 18 h was necessary for its full proliferative effect. However, a significant increase in proliferation was observed even with a few hours incubation, followed by removal of the agonist, suggesting that early intracellular events that follow muscarinic receptor activation are of most importance.

The effect of carbachol was compared with that of other neurotransmitters under basal conditions and in cells stimulated by serum. The proliferative effect of carbachol could not be detected in the presence of serum, which by itself stimulated astrocyte proliferation by about 12-fold. The effect of histamine was similar to that of carbachol, as its mitogenic effect was seen only in the absence of serum. Histamine had been shown to be mitogenic in a number of cell types, and its effect is believed to be mediated by activation of histamine H_1 and/or H_2 receptors (Van der Ven et al., 1993). Norepinephrine and GABA had no effect on astrocyte proliferation under either experimental condition. On the other hand, glutamate did not affect basal proliferation of astrocytes, but significantly inhibited serum-induced proliferation. This finding confirms a previous observation by Condorelli et al. (1989), also in rat cortical astrocytes. Finally, serotonin inhibited both basal and serum-stimulated astrocyte proliferation. It is important to consider that several of these neurotransmitters stimulate the metabolism of phosphoinositides in astrocytes (Hansson et al., 1987; Milani et al., 1989; Enkvist et al., 1989). Thus, it appears that events that follow activation of phospholipase C, and possibly also mobilization of Ca^{2+} and activation of protein kinase C, may be relevant for proliferation and are differentially activated by neurotransmitters. The study of activation of immediate-early-genes (e.g. *c-fos*, *c-myc*) and other genes may be a possible area for further fruitful investigations. Furthermore, the formation of other early stage second messengers (e.g. inositol 1,3,4,5-tetraphosphate, phosphatidic acid) may also be differentially affected by these neurotransmitters.

Phorbol esters, activators of protein kinase C, have been shown to cause proliferation of astrocytes (Murphy et al., 1987) and this was confirmed in the present study and extended to astrocytoma cells. The mitogenic effect of insulin-like growth factor-I (IGF-I) and of prolactin in

astrocytes had been shown to involve activation of protein kinase C (Tranque et al., 1992; De Vito et al., 1993). As muscarinic m3 and m5 receptors are expected to cause activation of protein kinase C, via diacylglycerol, we investigated whether inhibition of this intracellular pathway would affect carbachol-induced proliferation of astrocytes and astrocytoma cells. The protein kinase C inhibitor H7 caused a concentration-dependent inhibition of basal proliferation, and also inhibited TPA- and carbachol-induced proliferation. As H7 is not a highly specific protein kinase C inhibitor, further experiments were carried out in cells where protein kinase C had been down-regulated by pretreatment with a relatively high concentration of TPA (Mudd and Raizada, 1990; Tranque et al., 1992). Under this experimental condition, the mitogenic effects of both TPA and carbachol were almost completely blocked. Altogether, these experiments provide initial evidence of an involvement of protein kinase C in the mitogenic effect of muscarinic receptor agonists. In addition to phosphoinositide metabolism, muscarinic receptor activation in astrocytes also causes hydrolysis of phosphatidylcholine (Gustavsson et al., 1993). Both pathways generate diacylglycerol, but most would derive from phosphatidylcholine hydrolyzed by phospholipase D, and would be expected to be involved in long term cellular effects such as proliferation. Among the several isozymes of protein kinase C, the α subtype is the most predominant form in astrocytes (Misra-Press et al., 1992). Differently from neuronal cells, most (90%) of protein kinase C in astrocytes is found in the cytosol; as most of this activity can be translocated to membranes, astrocytes may thus be particularly suited to respond to membrane phospholipid-linked receptors (Neary et al., 1988). Additionally, astrocytoma cells, and gliomas in general, express very high levels of protein kinase C (Couldwell et al., 1991), which may also be involved in the larger proliferative response to muscarinic receptor activation of astrocytomas compared to astrocytes.

Though activation of muscarinic receptors caused proliferation of glial cells, this action cannot be generalized to all muscarinic receptor subtypes or to all cell types. Indeed, in A9L cells and Chinese hamster ovary cells transfected with the muscarinic m1 receptor, carbachol was found to inhibit proliferation by a mechanism independent of protein kinase C activation (Baumgold and Dyer, 1994). Furthermore, activation of muscarinic m3 receptors in small cell lung carcinoma cells has been shown to inhibit DNA synthesis induced by serum, insulin and IGF-I (Williams and Lennon, 1991). On the other hand, it has been shown that cloned muscarinic m1, m3 and m5 receptor subtypes can act as agonist-dependent oncogenes, as their activation in transfected NIH 3T3 cells caused the appearance of foci of transformation which appears to be mediated by a *ras*-dependent signalling pathway (Gutkind et al., 1991; Mattingly et al., 1994).

In summary, our findings indicate that muscarinic receptor agonists act as mitogens in rat astrocytes. These results are of interest and may be relevant with respect to the role of acetylcholine and muscarinic receptors in brain development (Costa, 1993). It is tempting to speculate that this neurotransmitter, by activating those receptor subtypes that are coupled to phospholipid hydrolysis, and whose effect is enhanced in the neonatal rat brain (Balduini et al., 1987), would play a role in the proliferation of astrocytes during the brain growth spurt. The mitogenic effect of acetylcholine in human astrocytoma cells may be of relevance for the biology and therapeutics of this tumor, which is a common brain tumor, particularly among children.

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