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Differences in the Stimulation of the Phosphoinositide Cycle by Amine Neurotransmitters in Cultured Rat Forebrain Neurones and Astrocytes

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ABSTRACT. In this study, we compared the stimulation by carbachol (CCh), noradrenaline (NA), and histamine (HA) of phosphoinositide hydrolysis in rat forebrain neuronal and glial cultures. When Ca^{2+} was omitted from the stimulation buffer (low μM extracellular Ca^{2+}), amine-induced [^{3}H]inositol phosphate accumulation was reduced to a higher extent in astrocytes (70–80% for CCh and NA and 100% for HA) than in neurones (around 50–60% for all the amines). Furthermore, guanosine 5′-[γ-thio]trisphosphate (GTP[S]) stimulation of phosphoinositidase C (PIC) in membranes was 5-fold higher in neurones than in astrocytes. These results indicate differences in the mechanism of PIC stimulation in the two cell types. After 30 min stimulation in the presence of 10 mM Li^+, a higher accumulation of [^{3}H]inositol 4-monophosphate and [^{3}H]inositol 1,4-bisphosphate than of [^{3}H]inositol 1/3-monophosphate occurred for all agonists in neurones, whereas the opposite was observed in astrocytes. Moreover, in these cells stimulation for 5 min in the absence of Li^+ produced a 2–3-fold accumulation of all metabolites of the 3-kinase pathway of inositol-1,4,5-trisphosphate metabolism but not of those of the 5-phosphatase pathway. Thus, regardless of the amine receptor stimulated, the 3-kinase route appeared to prevail in astrocytes and the 5-phosphatase pathway in neurones. The histamine response in neurones differed from that of the other agonists in that it rapidly declined. Taken together these results indicate that the heterogeneity in amine stimulation of the phosphoinositide cycle previously observed in brain slices could arise to a great extent from the cellular diversity of this preparation and be related to the differential contribution of the amine receptors located in neurones and astrocytes. BIOCHEM PHARMACOL **54**;11: 1243–1251, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. astrocytes; neurones; inositol phosphates; amine neurotransmitters; Ca^{2+}

In the CNS many neurotransmitter receptors promote the hydrolysis of inositol phospholipids through activation of phosphoinositidase C (PIC)‡ with the generation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and *sn*-1,2-diacylglycerol [1, 2]. Stimulation of these cell-surface receptors also produces a transient Ca^{2+} rise that is independent of extracellular Ca^{2+} and due to $Ins(1,4,5)P_3$-induced mobilisation from intracellular stores, followed by a sustained phase of elevated intracellular Ca^{2+} which is attributed to entry across the plasma membrane through several possible mechanisms [3]. On the other hand, Ca^{2+} influx through ligand-gated or voltage-gated channels can result in enhanced PIC activity [4, 5], and K^+-induced depolarization has been shown to potentiate neurotransmitter-stimulated inositol phosphate accumulation, in a Ca^{2+}-dependent manner, in different CNS preparations [6, 7].

It is well established that there is heterogeneity in receptor stimulation of PIC activity, perhaps arising from the variety of isoforms of PIC and their different mechanism of activation. Three classes of PIC isoforms, termed β, γ and δ, have been identified. Only the β isoforms appear to be controlled by heterotrimeric G proteins (the α and/or βγ subunits), whereas the γ family is regulated by receptors with tyrosine kinase activity [8, 9]. Recent studies indicate that a δ isoform is regulated by both a G protein and Ca^{2+} [10]. In liver, PLC δ is coupled to α_1-adrenergic receptors through a new class of G protein, the transglutaminase II/G_h protein, that activates the enzyme in a Ca^{2+}-concentration-dependent manner [11]. In this context, several studies have described differences in the extracellular Ca^{2+} dependency of PIC stimulation by a variety of amine neurotransmitters in brain slices [12, 13] as well as in the ability of these agonists to stimulate PIC in brain membranes in the presence of GTP[S] [14–16]. There is also

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‡ *Abbreviations:* CCh, carbachol; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimal essential medium; FCS, foetal calf serum; GTP[S], guanosine 5′-[γ-thio]trisphosphate; HA, histamine; HS, horse serum; $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$, inositol mono-, bis-, tris- and tetrakisphosphate, respectively; InsPs, inositol phosphates; NA, noradrenaline; PIC, phosphoinositidase C; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol 4-phosphate; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate.

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evidence that the pattern of inositol phosphate isomers formed by muscarinic-receptor activation are different from those produced after Ca^{2+} entry [17]. Furthermore, Sarri et al. [16] demonstrated, in brain slices, different inositol phosphate isomeric profiles for two groups of agonist receptors: those that were able to induce phosphoinositide hydrolysis in membranes and those that were "indirectly" coupled to PIC.

Differences observed in brain slices or membranes for various agonists could depend on the cell type in which their receptors are located. Several studies have shown that amine receptor activation of phosphoinositide hydrolysis and increases in Ca^{2+} occur in both neuronal and glial cells [18, 19]. Some studies have reflected differences in PIC stimulation by amine receptors in neurones and astrocytes. We showed that in astrocyte-enriched primary cultures, the histamine H_1-receptor-mediated response was abolished at low extracellular Ca^{2+} [20], while the response in neurones was partially inhibited [21]. However, a partial Ca^{2+} dependency was reported by Pearce and Murphy [18] for the response to carbachol and noradrenaline in astrocytes. Furthermore, Wilson et al. [22] demonstrated higher α_1-adrenergic-receptor-induced [^{3}H]inositol phosphate ([^{3}H]InsPs) accumulation in astrocytes than in neurones, although receptor density was higher in neurones, and suggested differential coupling mechanisms for this receptor in the two cell types. In this work, we have used astrocyte-enriched and neuronal primary cultures from rat forebrain to compare the extracellular Ca^{2+} dependency of stimulated phosphoinositide hydrolysis, the guanine nucleotide activation of PIC, and the inositol phosphate isomeric profile for carbachol (CCh)-, histamine (HA)- and noradrenaline (NA)-receptor activation in order to investigate if differences observed in slices could be related to different receptor-PIC coupling or to different cellular receptor localisation.

MATERIALS AND METHODS

Materials

D-*myo*-[^{3}H]inositol (18.3 Ci/mmol) was obtained from Amersham (Little Chalfont, UK); Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM) and foetal calf serum (FCS) were from Flow Laboratories; horse serum (HS) from GIBCO; penicillin G, streptomycin sulphate, insulin, progesterone, putrescine, transferrin, cytosine arabinoside and HA, CCh, NA from Sigma; guanosine 5′-[γ-thio]trisphosphate (GTP[S]) from Boehringer Mannheim; Dowex 1-X8 from BioRad.

Cultures

Astrocyte-enriched and neuronal cultures were prepared as previously described [23]. Briefly, for astrocyte-enriched cultures brain hemispheres of newborn Sprague–Dawley rats were dissociated into single cells by successive passages through nylon cloths of 211- and 135-μm mesh openings. Cells were suspended in culture medium (90% DMEM, 10% FCS, 20 U/mL penicillin, and 20 μg/mL streptomycin) to a final density of 0.6×10^6 viable cells/mL. Two milliliters of the suspension was seeded onto 35-mm diameter plastic Petri dishes and incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2. Culture medium was renewed once a week and confluent monolayers were used after 15–20 days in culture.

Forebrain neuronal primary cultures were prepared from 16-day-old rat embryos. Brain hemispheres were dissociated by successive passages through nylon cloths of 135- and 22-μm mesh openings and cells were suspended in 90% DMEM, 10% HS, 20 U/mL penicillin, and 20 μg/mL streptomycin. Two milliliters of the cell suspension (0.6×10^6 viable cells/mL) was seeded onto 35-mm diameter plastic Petri dishes previously coated with poly-L-α-ornithine (0.01% w/v). After 1 day in culture, the initial culture medium was replaced by serum-free glia-conditioned medium supplemented with insulin (5 μg/mL), transferrin (100 μg/mL), putrescine (100 μM), progesterone (20 nM), and Na_2SeO_3 (30 nM). Two days later cytosine arabinoside (5 μM final concentration) was added, and after 24 hr the medium was replaced by serum-free hormone-supplemented glial-conditioned medium. Neuronal cultures were incubated under the same conditions referred to above for astrocytes and were used after 7–8 days in culture.

Indirect immunocytochemical studies indicated that less than 5% of the cells present in the astrocyte-enriched cultures were neurone-specific enolase (NSE) positive, while more than 95% of the cells present in the neuronal primary cultures were NSE positive. Although the majority of cells in the astrocyte-enriched cultures were GFAP positive, there was a significant contamination with microglial cells as demonstrated by the histochemical reaction for nucleoside diphosphatase (NDPase). However, reduction of microglial cell contamination with cytosine arabinoside (5 μM) [24] had no effect in amine-induced [^{3}H]InsPs accumulation in glial primary cultures (not shown).

Phosphoinositide Hydrolysis

INTACT CELLS. Phosphoinositide breakdown was monitored by measuring the formation of [^{3}H]inositol phosphates in cells prelabelled for 24 hr with 1 μCi of [^{3}H]inositol in 1 mL of EMEM hormone-supplemented conditioned medium. A steady-state incorporation of label was reached by 18–20 hr in both cell cultures. Afterwards, the cultures were washed with a Krebs-HEPES buffer (in mM: NaCl 106, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 10, HEPES 20, LiCl 10, pH 7.4) and preincubated for 15 min in 1 mL of the same buffer before adding the agonists at maximally effective concentrations [18–22]. After 30 min stimulation, the medium was replaced by 2 mL of cold methanol-0.12 N HCl (1:1 v/v) and the dishes left at 4° for 1 hr. Aliquots of 1.5 mL of the extracts were diluted with 6 mL of water and passed through columns of 0.5 mL Dowex AG-1-X8 (formate form) resin [14]. To

determine tritium incorporated into lipids, the methanol-HCl insoluble residue was dissolved in 2 mL of 5% Triton X-100 and 0.2 mL aliquots counted. Experiments were performed in triplicate. For determination of isomeric [^{3}H]InsPs by HPLC, cells were labelled with 1–4 μCi/mL of [^{3}H]inositol and triplicate extracts were pooled. After filtration through 0.2-μm filters, samples were injected to a Merck-Hitachi (IGODA S.A.) HPLC system equipped with a Whatman Partisil 10 SAX column and a Whatman CSK I guard column. The [^{3}H]InsPs isomers were separated with an ammonium phosphate (adjusted to pH 3.7 with phosphoric acid) gradient [25] and identified by comparison to authentic [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 and breakdown products of [^{3}H]Ins(1,3,4)P_3.

MEMBRANES. Cultures of astrocytes and neurones were washed with fresh medium and the cells scrapped and homogenised in 10 vol. of ice-cold 20 mM Tris/HCl/1 mM EGTA, pH 7.0 by using a motor-driven Dounce homogeniser with a Teflon pestle (20 strokes at 800 rpm). Homogenates were centrifuged for 15 min at 39,000 × g; pellets were washed three times and stored at −80° until required for use. Membranes were labelled with [^{3}H]inositol (8–10 μCi/mL) in the presence of 1 mM CMP as previously described [26]. After the labelling period, membranes were centrifuged and the pellets obtained were resuspended by sonication in 25 mM Tris/maleate, pH 6.8. Aliquots of resuspended membranes (150 μg of protein) were incubated for 15 min at 37° in 100 μL of the same buffer containing (in mM): $MgCl_2$ 6, ATP 2, EGTA 3, $CaCl_2$ 0.7 (300 nM free Ca^{2+}) and, when present, GTP[S]. Incubations were stopped by addition of 1.2 mL of chloroform/methanol (1:2, v/v). A 0.5 mL portion of both 0.25 M HCl and chloroform were added to the tubes and the phases were separated. A 1 mL portion of the upper phase was neutralised with 1.5 M NH_4OH, diluted with 4 mL of distilled water and passed through the Dowex columns.

RESULTS

The accumulation of [^{3}H]$InsP_1$, [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$ induced by CCh, NA and HA was studied in [^{3}H]inositol prelabelled neurone and astrocyte-enriched cultures, after stimulation with agonists for 30 min in the presence of 10 mM Li^+. Incorporation of label into phosphoinositides in neurones was 13% of added [^{3}H]inositol, but was only 6% in astrocytes. As expected under these conditions, [^{3}H]$InsP_1$ accumulated in a larger amount than [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$ levels were very low or nonsignificant, both under basal and stimulated conditions (Fig. 1). In neurones (Fig. 1A), CCh was the most effective agonist (311%, 319% and 75% over basal accumulation for [^{3}H]$InsP_1$, [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$, respectively) followed by NA (152%, 145% for [^{3}H]$InsP_1$ and [^{3}H]$InsP_2$, respectively) and HA, which elicited significant accumulation only of [^{3}H]$InsP_1$ (100%). In astrocytes (Fig. 1B), CCh and NA were equally effective and gave a higher percent

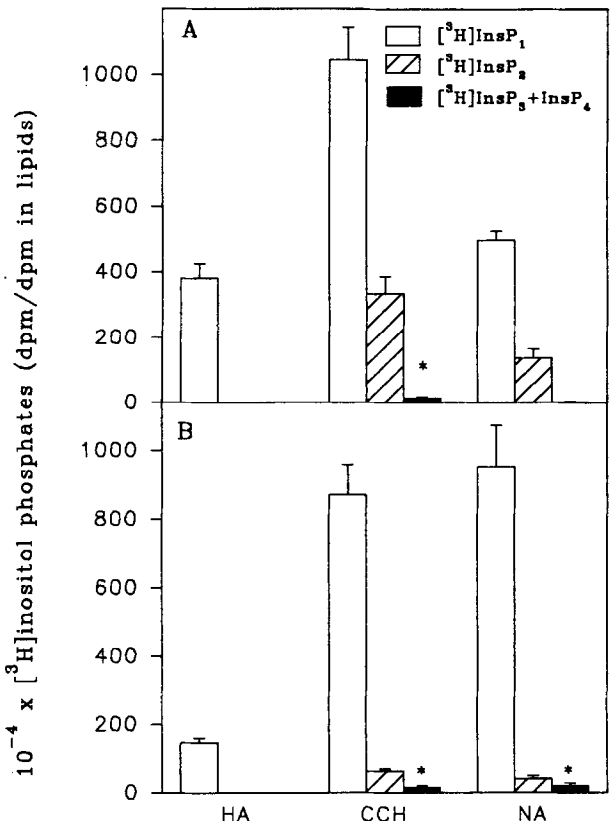

FIG. 1. Accumulation of [^{3}H]inositol phosphates induced by HA, CCh and NA in neuronal and glial cultures. Cells prelabelled with [^{3}H]inositol (1 μCi/mL) were incubated for 30 min in the absence (basal) or presence of HA (100 μM), CCh (1 mM) or NA (50 μM) in Krebs-HEPES buffer in the presence of 2.5 mM Ca^{2+} and 10 mM Li^+. Reactions were stopped and [^{3}H]inositol phosphates separated by Dowex anion-exchange chromatography as described in "Materials and Methods." (A) Neurones, (B) astrocytes. Results are dpm − dpm in basal (in neurones: 377 ± 44, 110 ± 13, 19 ± 2; in astrocytes: 108 ± 10, 49 ± 3, 36 ± 6 dpm × 10^{-4}/dpm in lipids for [^{3}H]$InsP_1$, [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$, respectively) and are means ± SEM from 9–11 experiments performed in triplicate using different cell preparations. *, Indicates significantly different from basal (Student's *t*-test, $P < 0.05$).

stimulation than in neurones for [^{3}H]$InsP_1$, but not for [^{3}H]$InsP_2$ (805 and 124% for CCh; 880 and 82% for NA, respectively). Thus, a larger percent accumulation of [^{3}H]$InsP_2$ relative to [^{3}H]$InsP_1$ was always detected in neurones (16–38%) compared to astrocytes (4–7%). CCh and NA significantly stimulated accumulation of [^{3}H]$InsP_3$ + $InsP_4$ (44 and 58%) in astrocytes. In these cells, the efficacy of HA was slightly higher than in neurones (151%) and again, no significant [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$ accumulation was observed.

We examined the dependence on extracellular Ca^{2+} of the agonist responses by incubating the cells in buffer with 0 to 4 mM added Ca^{2+}. Results for accumulation of [^{3}H]$InsP_1$ and [^{3}H]$InsP_2$ are shown in Fig. 2. In both cell types, no significant differences in responses were observed when extracellular Ca^{2+} was increased from 1 to 4 mM

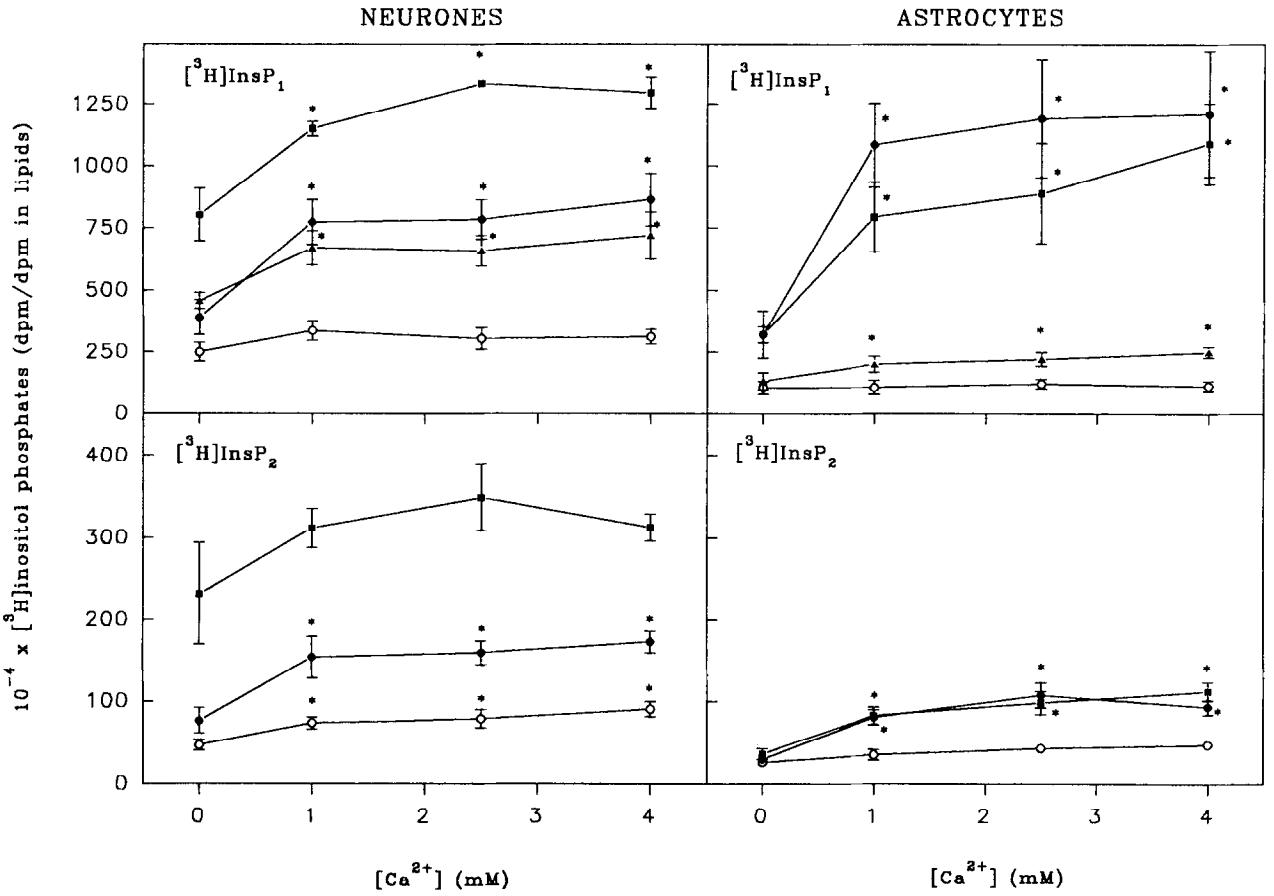

FIG. 2. Effect of extracellular Ca^{2+} on the HA-, CCh- and NA-induced $[^3H]InsP_1$ and $[^3H]InsP_2$ accumulation in neurones and astrocytes. Cells prelabelled with $[^3H]$inositol (1 μCi/mL) were incubated for 30 min in the absence (basal, ○) or presence of 100 μM HA (▲), 1 mM CCh (■) or 50 μM NA (●) in Krebs–HEPES buffer containing increasing concentrations of added Ca^{2+}. Reactions were stopped and $[^3H]$inositol phosphates separated by Dowex anion-exchange chromatography. Results are means ± SEM from 3–5 experiments. *, Indicates significantly different from 0 mM Ca^{2+} under each condition (ANOVA, Duncan test, $P < 0.05$).

(ANOVA, Duncan test). However, when no Ca^{2+} was added to the medium (nominally Ca^{2+}-free buffer, ≈10 μM Ca^{2+} remaining in Krebs buffer according to Kendall and Nahorski [12]), agonist responses appeared to decrease to a higher extent in astrocytes (Fig. 2). In 7 different culture preparations, $[^3H]InsP_1$ accumulations induced by CCh, NA and HA in nominally Ca^{2+}-free buffer in neurones were 50 ± 8%, 39 ± 8% and 51 ± 9% of those induced at 2.5 mM Ca^{2+} (concentration routinely used). A similar reduction occurred in $[^3H]InsP_2$ accumulations induced by CCh and NA (58 ± 12% and 43 ± 11% of the effect at 2.5 mM Ca^{2+}, respectively). However, in astrocytes in nominally Ca^{2+}-free buffer only 28 ± 5 and 22 ± 4% of the $[^3H]InsP_1$ responses to CCh and NA, respectively remained, and the effect of HA was abolished. No significant accumulation of $[^3H]InsP_2$ was observed in astrocytes under this condition. Calcium required for part of the response to CCh and NA in neurones may enter the cell through voltage-dependent L-type Ca^{2+} channels, since verapamil (10 μM) reduced the CCh and NA-induced $[^3H]InsP_1$ accumulation by ca. 40%. However, as the Ca^{2+} channel blocker did not affect the response to HA in neurones or to any of the agonists in astrocytes (Fig. 3), a different mechanism for Ca^{2+} entry must have been operative in these cases.

To investigate if the different extracellular Ca^{2+} dependency of the responses was related to the direct or indirect coupling of the receptors to PIC, we examined the ability of GTP[S] to stimulate $[^3H]$inositol phosphate formation in cell membranes prelabelled with $[^3H]$inositol. In neurones, GTP[S] (1 μM) produced a 5-fold higher stimulation of $[^3H]InsP_2$ accumulation than in astrocytes (1033 ± 195% of basal, N = 8 and 200 ± 22% of basal, N = 7, respectively). No significant accumulation of $[^3H]InsP_1$ or $[^3H]InsP_3$ was observed. The potency of the guanine nucleotide stimulating $[^3H]InsP_2$ formation, estimated from concentration-effect curves, was similar in both cell types (24 ± 2 and 17 ± 3 nM in neurones and astrocytes, respectively; N = 2). GTP[S] was also more effective in stimulating PIC in permeabilized neurones than in astrocytes (1.3-fold) (not shown).

Metabolism of $Ins(1,4,5)P_3$ can occur via the 5-phosphatase and 3-kinase routes, giving rise to different InsPs isomers after agonist stimulation [27]. Studies in brain slices

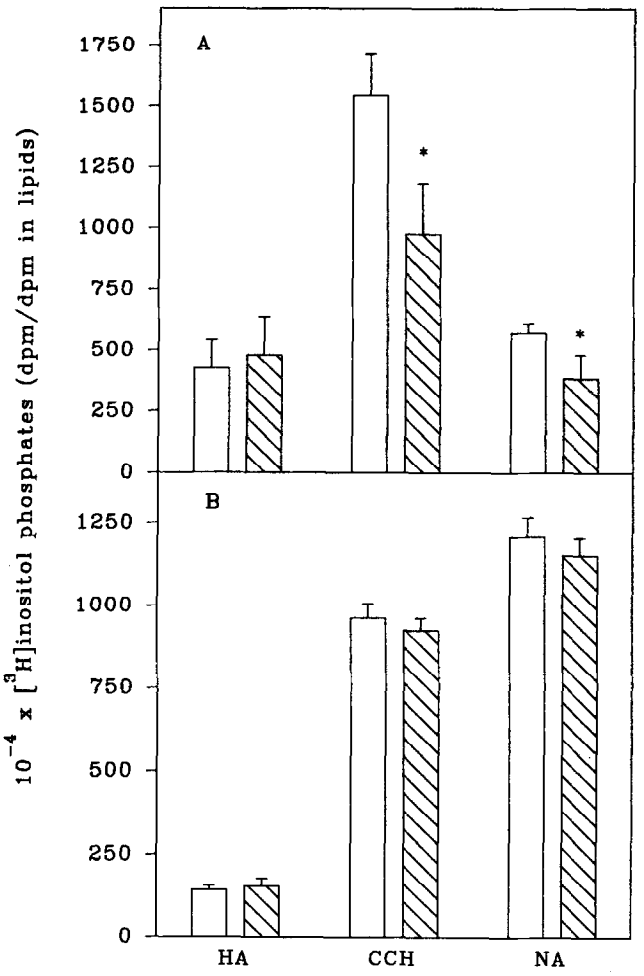

FIG. 3. Effect of verapamil in [^{3}H]inositol phosphate formation induced by CCh and HA in neurones (A) and astrocytes (B). Cells prelabelled with [^{3}H]inositol (1 μCi/mL) were incubated for 15 min in the absence (open bars) or presence of 10 μM verapamil (hatched bars) before the addition of HA (100 μM), CCh (1 mM) or NA (50 μM) in Krebs–HEPES buffer containing 2.5 mM Ca^{2+} and 10 mM Li^+. Reactions were stopped and [^{3}H]inositol phosphates separated by Dowex anion-exchange chromatography as described in "Materials and Methods." Results are dpm minus dpm in basal (in neurones: 497 ± 72, 472 ± 72; in astrocytes: 98 ± 15, 89 ± 17, dpm × 10^{-4}/dpm in lipids in the absence or presence of verapamil, respectively) and are means ± SEM from 3–4 experiments in neurones and means ± SE in a representative experiment that was replicated with similar results in astrocytes. *Indicates significantly different from the response in the absence of verapamil (Student's *t*-test, $P < 0.05$).

and membranes have suggested that different profiles of [^{3}H]InsPs isomers are related to the mode of receptor activation of PIC; guanine nucleotide-dependent activation in membranes or indirect activation probably through induction of Ca^{2+} entry [16]. To test if a similar relationship could be established in neurones and in astrocytes, we separated by HPLC the isomers of the more abundant [^{3}H]InsPs accumulated ([^{3}H]$InsP_1$ and [^{3}H]$InsP_2$) after agonist stimulation for 30 min in the presence of Li^+ in both cell types. Table 1 shows that, in neurones, the major [^{3}H]$InsP_1$ isomers produced in response to all three agonists were [^{3}H]Ins(1/3)P and [^{3}H]Ins(4)P, which accumulated in a similar percentage. For CCh and NA, there was also a lower accumulation of [^{3}H]Ins(1,4)P_2 (15–20% with respect to total [^{3}H]InsPs measured). In astrocytes, however, all agonists induced a larger percent accumulation of [^{3}H]Ins(1/3)P than of [^{3}H]Ins(4)P (70 and 28% of total [^{3}H]InsPs, respectively), and for CCh and NA there was a very small accumulation of [^{3}H]Ins(1,4)P_2 (1–2%). A small or no significant accumulation of [^{3}H]Ins(1,3)P_2 and [^{3}H]Ins(3,4)P_2 was found under these conditions. In order to compare the profile of [^{3}H]InsPs accumulated after agonist stimulation in neurones and astrocytes, we calculated the ratio between [^{3}H]Ins(4)P + [^{3}H]Ins(1,4)P_2/[^{3}H]Ins(1/3)P + [^{3}H]Ins(1,3)P_2 + [^{3}H]Ins(3,4)P_2. This can be taken as an index of the relative importance of the 5-phosphatase and 3-kinase pathways of Ins(1,4,5)P_3 metabolism independently of the efficacy of each agonist and of differences in basal levels in both cell types. Nevertheless, this index is just an approximation, since in intact cells some [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(4)P can originate from PtdInsP hydrolysis [28] and some [^{3}H]Ins(1,4)P_2 can be degraded to [^{3}H]Ins(1)P [29]. Table 2 shows the ratios calculated from the data in Table 1. As can be observed, values are quite different for the two cell types. In neurones, an index near 1 was obtained under basal conditions but after agonist stimulation, accumulation of 5-phosphatase metabolites nearly doubled that of the 3-kinase products. Conversely, in astrocytes, both basal and agonist-stimulated indexes reflected that the 5-phosphatase route metabolites were less than half those of the 3-kinase pathway. A firmer demonstration that the pattern of inositol phosphate isomers observed in astrocytes reflects a higher activation of the 3-kinase pathway was obtained by analysing all the InsPs isomers formed after stimulation for 5 min in the absence of lithium. Results for the more efficacious agonists CCh and NA are shown in Table 3. Both agonists induced a 2–3-fold stimulation over basal in the accumulation of the 3-kinase metabolites [^{3}H]Ins(1/3)P, [^{3}H]Ins(1,3)P_2, [^{3}H]Ins(3,4)P_2, [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4. Only in the case of NA was a significant accumulation of the 5-phosphatase route metabolite [^{3}H]Ins(4)P (66% of basal) observed.

A striking feature of the HPLC results at 30 min was that HA had no significant effect on [^{3}H]Ins(1,4)P_2 accumulation, although it was able to induce a significant accumulation of [^{3}H]Ins(4)P that can only arise from hydrolysis of [^{3}H]Ins(1,4)P_2. Since it is possible that, even though lithium was present in these cells, a rapid degradation of [^{3}H]Ins(1,4)P_2 occurred, we investigated the kinetics of [^{3}H]inositol phosphate accumulation in neurones and astrocytes in response to HA and CCh. In neurones (Fig. 4A), there was similar [^{3}H]InsPs accumulation up to 2.5 min after stimulation with both agonists. However, a rapid decline in HA-induced [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$ formation was found thereafter, while for CCh both [^{3}H]InsPs increased further up to 5 min and remained at

TABLE 1. Accumulation of [^{3}H]inositol phosphate isomers induced by HA, CCh and NA in neurones and astrocytes

	[^{3}H]Inositol phosphates (dpm)		
	HA	CCh	NA
Neurones			
[^{3}H]Ins(1/3)P	3104 ± 893	23776 ± 3945	6888 ± 1368
[^{3}H]Ins(4)P	4555 ± 1022	27079 ± 2881	10238 ± 737
[^{3}H]Ins(1,3)P_2	7 ± 7*	117 ± 35	6 ± 6*
[^{3}H]Ins(1,4)P_2	381 ± 160*	11803 ± 3809	2709 ± 396
[^{3}H]Ins(3,4)P_2	35 ± 20*	94 ± 37	47 ± 35*
Astrocytes			
[^{3}H]Ins(1/3)P	3037 ± 597	27271 ± 1042	38831 ± 8677
[^{3}H]Ins(4)P	1166 ± 214	10790 ± 729	12385 ± 5076
[^{3}H]Ins(1,3)P_2	17 ± 10*	62 ± 20	178 ± 37
[^{3}H]Ins(1,4)P_2	467 ± 467*	440 ± 132	826 ± 100
[^{3}H]Ins(3,4)P_2	18 ± 18*	25 ± 13*	164 ± 25

Cells prelabelled with [^{3}H]inositol (1 and 4 μCi/mL for neurones and astrocytes, respectively) were incubated for 30 min in the absence (basal) or presence of HA (100 μM), CCh (1 mM) or NA (50 μM) in Krebs-HEPES buffer in the presence of 2.5 mM Ca^{2+}. Reactions were stopped and [^{3}H]inositol phosphates separated by HPLC as described in "Materials and Methods." Results shown are dpm − dpm in basal (in neurones: 12532 ± 1776, 10243 ± 2059, 17 ± 7, 1694 ± 530, 98 ± 38; in astrocytes: 4009 ± 1269, 1122 ± 515, n.d., 896 ± 232, 83 ± 7) and are means ± SEM for 3–4 experiments in neurones and 2–4 experiments in astrocytes.

* Indicates not significantly different from basal (paired Student's *t*-test, $P < 0.05$).

that level until at least 30 min. The kinetics of [^{3}H]InsP_1 accumulation were also different for HA and CCh. Maximum levels of [^{3}H]InsP_1 were reached by 10 min in response to HA, remaining constant thereafter, while continually increasing in response to CCh. In astrocytes (Fig. 4B), the amount of [^{3}H]InsP_2 and [^{3}H]InsP_3 + InsP_4 generated in response to HA at early times was significantly different from basal but smaller than in neurones and also declined, although with a slower time-course. In astrocytes, [^{3}H]InsP_2 and [^{3}H]InsP_3 + InsP_4 levels produced in response to CCh were much higher than for HA and declined after the first 5 min but were still significant at 30 min.

DISCUSSION

In the present study, we have examined the phosphoinositide breakdown induced by different amine neurotransmitters in neuronal and astrocyte-enriched cultures in order to compare its functionality in both cell types. Phosphoinositide turnover appears to be more active in neurones, since both [^{3}H]inositol incorporation into lipids and basal [^{3}H]InsPs accumulation were higher in these cells. Since neurones in culture develop neurites and form mature synapses [30], their basal electrical activity may accelerate phosphoinositide turnover. When cells were incubated with agonists for 30 min in the presence of Li^+, percent [^{3}H]InsPs accumulation induced by CCh and NA was higher in glial cells, whereas the HA effect was similar in both cell types. The rank order of agonist-induced [^{3}H]InsPs accumulation was CCh > NA > HA in neurones and CCh = NA > HA in astrocytes, in agreement with previous reports [19, 31].

TABLE 2. Indexes of the different metabolic pathways of [^{3}H]inositol phosphate degradation in neurones and astrocytes upon stimulation with HA, CCh and NA

	[^{3}H]Ins(4)P + [^{3}H]Ins(1,4)P_2/ [^{3}H]Ins(1/3)P + [^{3}H]Ins(1,3)P_2 + [^{3}H]Ins(3,4)P_2	
	Neurones	Astrocytes
No addition	0.92 ± 0.11	0.48 ± 0.03
HA	1.69 ± 0.21	0.39 ± 0.07
CCh	1.65 ± 0.08	0.42 ± 0.04
NA	1.97 ± 0.26	0.34 ± 0.07

Indexes were calculated from data shown in Table 1. Results are means ± SEM.

When the effect of raising extracellular Ca^{2+} was investigated, we observed that in both cells none of the amine responses was affected by elevations of extracellular Ca^{2+} from 1 to 4 mM. Similar results have been reported for CCh and HA stimulation of phosphoinositide hydrolysis in U373 astrocytoma, HeLa cells and guinea-pig cortical and cerebellar slices, but not for HA and NA responses in rat and mouse cerebral cortex, where a dramatic enhancement was observed when extracellular Ca^{2+} was raised to 4 mM [32, 13]. The physiological relevance of these effects at high mM Ca^{2+} and the reason for the discrepancies with our results are not clear. Differences in Ca^{2+} dependency for the effects of agonists in neurones and astrocytes were observed in experiments performed in nominally Ca^{2+}-free buffer. In this situation, [^{3}H]InsP_1 and [^{3}H]InsP_2 accumulations in response to all agonists were diminished by ca. 50–60% in neurones, whereas in astrocytes [^{3}H]InsP_1 accumulation was decreased 70–80% in response to CCh and NA and abolished for HA. Furthermore, no [^{3}H]InsP_2 accumulation was observed. Higher extracellular Ca^{2+} dependency in astrocytes than in neurones was also observed for muscarinic receptor-induced intracellular Ca^{2+} increases [33, 34]. In slices, different studies [12, 13] found that in the absence of added Ca^{2+} a significant % of the

TABLE 3. Accumulation of [^{3}H]InsPs isomers induced by CCh and NA in astrocytes in the absence of lithium

	[^{3}H]Inositol phosphates (dpm)		
	Basal	CCh	NA
[^{3}H]Ins(1/3)P	967 ± 65	3934 ± 924*	3749 ± 165*
[^{3}H]Ins(4)P	2177 ± 219	2399 ± 334	3285 ± 145*
[^{3}H]Ins(1,4)P_2	3078 ± 382	3280 ± 170	2950 ± 229
[^{3}H]Ins(3,4)P_2	384 ± 15	992 ± 108*	1092 ± 109*
[^{3}H]Ins(1,3,4)P_3	391 ± 107	843 ± 79*	903 ± 154*
[^{3}H]Ins(1,4,5)P_3	1019 ± 197	1244 ± 95	1051 ± 241
[^{3}H]Ins(1,3,4,5)P_4	517 ± 168	1318 ± 215*	1330 ± 433*

Cells prelabelled with [^{3}H]inositol (2 μCi/mL) were incubated for 5 min in the absence (basal) or presence of CCh (1 mM) or NA (100 μM) in Krebs-HEPES buffer in the absence of lithium. Reactions were stopped and [^{3}H]inositol phosphates separated by HPLC as described in the "Materials and Methods." Results are means ± SEM for 3 experiments.

* Indicates significantly different from basal.

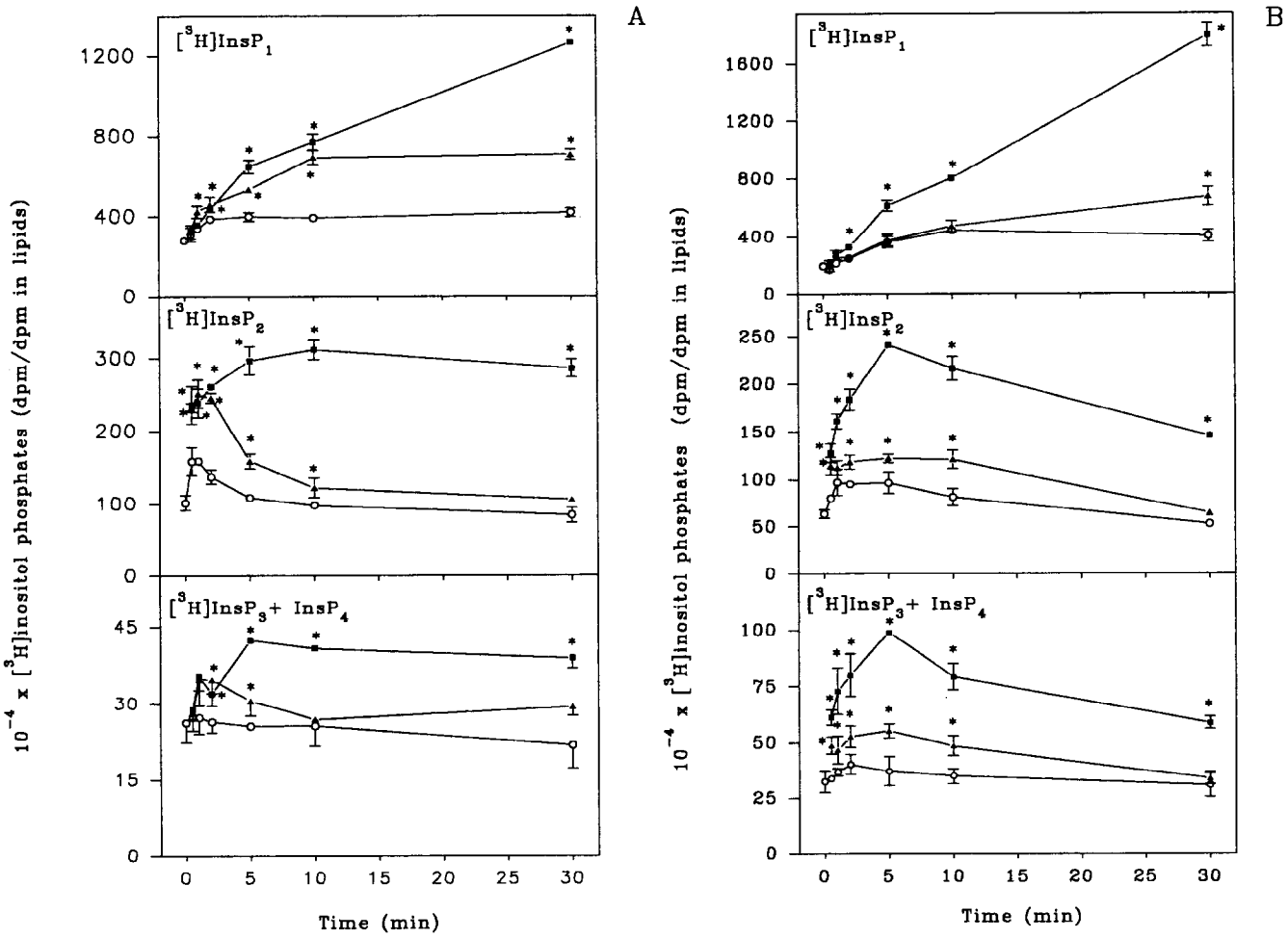

FIG. 4. Time-course of [^{3}H]inositol phosphate formation induced by CCh and HA in neurones (A) and astrocytes (B). Cells prelabelled with [^{3}H]inositol (3 and 5 μCi/mL for neurones and astrocytes, respectively) were incubated for the indicated times in the absence (basal, ○) or presence of 100 μM HA (▲) or 1 mM CCh (■) in Krebs–HEPES containing 2.5 mM Ca^{2+}. Reactions were stopped and [^{3}H]inositol phosphates separated by Dowex anion-exchange chromatography as described in "Materials and Methods." Results are means ± SE from triplicate determinations in a representative experiment that was replicated with similar results. *Indicates significantly different from basal (Student's *t*-test, $P < 0.05$).

response to CCh and NA remained unaffected, whereas that of HA was abolished. Thus, our results suggest that astrocyte H_1-receptors largely contribute to the HA effect observed in slices.

In neurones, apart from a lower extracellular Ca^{2+} dependency of the agonist effects, we found a more pronounced activation of PIC by GTP[S] in membranes in comparison to astrocytes. As occurred in brain membranes [26], no [^{3}H]$InsP_3$ accumulation was observed in membranes of the cells, probably due to its rapid degradation to [^{3}H]Ins(1,4)P_2 by the membrane-bound 5-phosphatase. In contrast, the 1-phosphatase is mostly soluble and thus [^{3}H]$InsP_2$ accumulated more than [^{3}H]$InsP_1$, which in this study did not reach significant levels. The potency of GTP[S] for stimulating [^{3}H]$InsP_2$ formation in membranes from both neurones and astrocytes was similar to that we previously reported in brain cortical membranes [26]. Thus, the same type of G protein may be activated in both cell types, but a smaller pool of this protein or of the PIC it regulates may exist in astrocytes.

It has been demonstrated that PIC isozymes are differentially expressed in neurones and astrocytes. PIC-β and -γ are more abundant in neurones and PIC-δ in glial cells [35]. Activation of PIC-β_1, which is partially membrane-bound, requires interaction with α subunits of the G_q family of heterotrimeric GTP-binding proteins [36]. Receptors coupled to these G proteins activate PIC-β at resting intracellular Ca^{2+} concentrations [14, 37]. In contrast, PIC-δ isoform activation by receptors apparently involves both G proteins (other than G_q) and Ca^{2+} [10]. Recently, it has been reported that the PIC-δ_1 isoform is regulated by a new class of G proteins, the transglutaminaseII/G_h proteins. The coupling of G_h with PIC-δ_1 is inhibited in the presence of EGTA, and the activation of the enzyme by GTP[S]-bound G_h is dependent on μM Ca^{2+} concentrations [11]. Thus, our results may reflect a predominant activation of a PIC-β in neurones and of PIC-δ in astrocytes, at least for muscarinic receptors.

Sarri et al. [16] suggested that two groups of receptors could be defined according to the mode of activation of PIC in rat brain slices. The classification was based upon the profile of [^{3}H]inositol phosphate isomers formed, the efficacy of the CDP-DAG response, the Ca^{2+} dependency, and the ability to stimulate PIC in cortical membranes in a GTP[S]-dependent manner. According to these parameters, CCh pertained to one group and NA and HA to another. In contrast with these results, we did not observe marked differences among agonists in the profile of [^{3}H]inositol phosphate isomers formed within each cell type, but a remarkable difference was observed between astrocytes and neurones, which indicated that the predominant pathway of [^{3}H]Ins(1,4,5)P_3 degradation is different in the two cells. In stimulated neurones, more [^{3}H]Ins(1,4,5)P_3 appears to be metabolised through the 5-phosphatase route because [^{3}H]Ins(4)P and [^{3}H]Ins(1,4)P_2 are the predominant $InsP_1$ and $InsP_2$ accumulated. Accordingly, Gray et al. [37] recently showed that upon muscarinic receptor activation, metabolism of [^{3}H]Ins(1,4,5)P_3 occurs predominantly via the 5-phosphatase route in rat cerebellar granule cells. However, in astrocytes, all the metabolites of the 3-kinase pathway were significantly increased after 5 min stimulation with CCh and NA in the absence of Li^+. Furthermore, [^{3}H]Ins(1/3)P was the isomer accumulated in a larger amount after 30 min stimulation with the three agonists in the presence of 10 mM Li^+. The fact that in astrocytes [^{3}H]Ins(1,4,5)P_3 degradation occurred predominantly via 3-kinase could explain the low levels of [^{3}H]$InsP_2$ detected in these cells, since degradation of $InsP_2$ isomers formed by successive dephosphorylation of [^{3}H]Ins(1,3,4,5)P_4 to Ins(1)P and Ins(3)P is less sensitive to lithium inhibition than that of [^{3}H]Ins(1,4)P_2 to [^{3}H]Ins(4)P [28]. A higher accumulation of the 3-kinase pathway metabolites has been reported in brain slices for CCh stimulation [38, 16] but not for NA or HA, agonists whose effects are more "Ca^{2+}-dependent" in that preparation. In contrast, we have observed a greater accumulation of [^{3}H]Ins(1,3,4,5)P_4 metabolites for all agonists in astrocytes, where the effects are more dependent on extracellular Ca^{2+} than in neurones. Agonist-induced Ca^{2+} entry could, apart from activating PIC, stimulate [^{3}H]Ins(1,4,5)P_3 3-kinase, which is a Ca^{2+}-calmodulin-dependent enzyme [39]. Thus, it is possible that glial cells significantly contribute to the CCh-induced accumulation of [^{3}H]Ins(1,3,4,5)P_4 observed in slice preparations.

Finally, the rapid decrease in [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ + $InsP_4$ accumulation observed after HA stimulation in neurones could indicate that there is a limited pool of $PtdInsP_2$ and/or PtdInsP available for H_1-receptor-stimulated PIC or that a rapid desensitisation of these receptors occurs in these cells, as has been observed previously in cerebellar granule cells [40] and human astrocytoma cells [41]. Little or no accumulation of the more phosphorylated inositols was also reported after HA stimulation for long periods in brain slices [42].

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