Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H₁-histamine receptors on human astrocytoma cells

Differential induction of desensitization by agonists

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Although activation of muscarinic cholinergic receptors on 1321N1 human astrocytoma cells results in a linear accumulation of inositol phosphates for up to 60 min in the presence of LiCl [Masters, Quinn & Brown (1985) *Mol. Pharmacol.* **27**, 325–332], activation of H₁-histamine receptors resulted in an increase in total inositol phosphate formation that was maintained for less than 5 min. The effects of stimulation of these two receptors on accumulation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃], inositol 1,3,4-trisphosphate [Ins(1,3,4)P₃] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄] were also examined. Incubation of 1321N1 cells with carbachol resulted in a rapid accumulation of all three inositol phosphates, reaching a maximum within 30 s; this elevated value was maintained for up to 60 min. The rate of disappearance of Ins(1,3,4)P₃ from carbachol-treated cells after the addition of atropine paralleled or exceeded the rate of disappearance of Ins(1,4,5)P₃. Although the initial rates of accumulation of Ins(1,4,5)P₃, Ins(1,3,4)P₃ and Ins(1,3,4,5)P₄ in the presence of histamine were similar to that observed with carbachol, the amounts of these inositol phosphates had returned to control values within 5 min after the addition of histamine. The results indicate that, although the acute effects of muscarinic receptor and H₁-histamine receptor stimulation on phosphoinositide hydrolysis are very similar, the histamine receptor is desensitized rapidly, whereas the muscarinic receptor is not. This effect on histamine-receptor function is apparently homologous, since preincubation of 1321N1 cells with histamine did not decrease the subsequent response to carbachol.

INTRODUCTION

Ca²⁺-mobilizing hormones activate a phospholipase C (Michell, 1975) that catalyses formation of the cellular second messengers Ins(1,4,5)P₃ (Berridge *et al.*, 1983; Berridge & Irvine, 1984) and diacylglycerol (Nishizuka, 1984) from polyphosphoinositides. Ins(1,4,5)P₃ apparently mobilizes Ca²⁺ from endoplasmic reticulum (Streb *et al.*, 1983; Berridge & Irvine, 1984), whereas diacylglycerol acts, in concert with Ca²⁺, as the endogenous activator of protein kinase C. Although this scheme of second-messenger action is in general supported by data obtained with a number of hormone receptors and many target tissues, neither the details of formation and metabolism of the phosphoinositide hydrolysis products nor identification of their intracellular sites of action has been fully established. Irvine *et al.* (1984, 1985) have reported that a second InsP isomer, Ins(1,3,4)P₃, accumulated in rat parotid gland after carbachol stimulation. However, the kinetics of appearance and disappearance of the two InsP₃ isomers were markedly different. That is, whereas the accumulation and degradation of Ins(1,4,5)P₃ were rapid, Ins(1,3,4)P₃ accumulated with a lag, eventually attained much higher concentrations than Ins(1,4,5)P₃, and dis-

appeared more slowly after cessation of hormonal challenge than did Ins(1,4,5)P₃ (Irvine *et al.*, 1985). Similar results have been reported by Burgess *et al.* (1985) for guinea-pig hepatocytes, pancreatic acinar cells and dimethyl sulphoxide-differentiated human myelomonocytic HL-60 leukaemia cells. The physiological importance of Ins(1,3,4)P₃ has not been defined. However, on the basis of its relatively slow rates of formation and disappearance, it has been proposed that this inositol phosphate does not subserve a role in the initial Ca²⁺-mobilizing effects of hormones.

The mechanisms of synthesis and degradation of Ins(1,3,4)P₃ also have not been defined fully. However, Batty *et al.* (1985) and Irvine *et al.* (1986) have reported the existence of Ins(1,3,4,5)P₄ in mammalian tissues. Ins(1,4,5)P₃ is apparently converted into Ins(1,3,4,5)P₄ by a specific Ins(1,4,5)P₃ 3-kinase (Irvine *et al.*, 1986), and the 5-dephosphorylation of this inositol tetrakisphosphate probably accounts for the occurrence of Ins(1,3,4)P₃. In most tissues the kinetics of accumulation of Ins(1,4,5)P₃ and Ins(1,3,4)P₃ after hormonal challenge are consistent with this hypothesis; however, extensive data in support of this proposal are not yet available.

1321N1 human astrocytoma cells have proved to be a

Abbreviations used: InsP₃, inositol trisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4)P₃, inositol 1,3,4-trisphosphate; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetrakisphosphate; Gro-PIIns, glycerophosphoinositide; InsP₁, inositol 1-phosphate; InsP₂, inositol 1,4-bisphosphate; Gro-PIInsP₂, glycerophosphoinositide 1,4-bisphosphate; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium.

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useful model for the study of receptor-mediated regulation of phosphoinositide turnover. Activation of muscarinic cholinergic (Masters *et al.*, 1984) and histamine (Nakahata *et al.*, 1986) receptors on these cells results in an increase in inositol phosphate formation, Ca^{2+} mobilization and cyclic AMP phosphodiesterase activity. Although the formation of InsP_3 in response to both cholinergic stimuli (Masters *et al.*, 1985a) and histamine (Nakahata *et al.*, 1986) has been documented in these cells, the relative contribution of the two InsP_3 isomers to this response has not been established. Furthermore, although the formation of total inositol phosphates is linear with time for 1 h in response to carbachol (Masters *et al.*, 1985a), in preliminary experiments we observed that the response to histamine was lost rapidly. In the current study we compare the formation of inositol phosphates in response to both muscarinic- and histamine-receptor stimulation.

MATERIALS AND METHODS

Materials

DMEM, EMEM, trypsin and fetal-calf serum were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Carbachol, histamine, atropine, ATP, ADP, AMP and ammonium formate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_3PO_4 (h.p.l.c. grade) was from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). *myo*- $[\text{^3H}]$ inositol was purchased from New England Nuclear (Boston, MA, U.S.A.) or American Radiolabeled Chemicals (St. Louis, MO, U.S.A.), and *myo*- $[\text{^3H}]$ inositol 1,4,5-trisphosphate (1 Ci/mmol) was obtained from Amersham. All chemicals and drugs were of reagent grade or the highest quality available.

Cell culture

1321N1 human astrocytoma cells were grown in 35 mm dishes with DMEM containing penicillin (50 units/ml) and streptomycin (50 mg/ml) as previously described (Meeker & Harden, 1982).

Analyses of inositol phosphates

The cells were labelled overnight with 1–10 μCi of $[\text{^3H}]$ inositol/ml of inositol-free DMEM. After labelling, the cells were washed twice with 1.5 ml of EMEM/Hepes (25 mM) and incubated in the same buffer for an additional 10 min. Agonists were added for the indicated times, and the reactions terminated by aspiration of the medium and addition of 0.5 ml of methanol (for total inositol phosphate assays) or 0.5 ml of 5% (v/v) trichloroacetic acid (for h.p.l.c. assays). The cells were scraped and rinsed with 0.5 ml of methanol (for assay of total inositol phosphates) or 0.5 ml of 5% trichloroacetic acid (for h.p.l.c. assays). Trichloroacetic acid extracts were centrifuged and the supernatants were stored at -20°C . For assay of total inositol phosphates, the pooled methanol fraction (1 ml) was combined with chloroform (1 ml) and water (0.9 ml). After centrifugation the upper phase was applied to a column containing 1 ml bed volume of resin (AG 1-X8; 100–200 mesh, formate form; Bio-Rad). Free inositol and glycerophosphate were eluted with 10 ml of water and 10 ml of 50 mM-ammonium formate respectively. Total inositol phosphates (InsP_1 , InsP_2 and InsP_3) then were eluted with 8 ml of 1.0 M-ammonium formate/100 mM-formic acid.

Preparation of ^{32}P -labelled $\text{Ins}(1,4,5)\text{P}_3$ from human erythrocyte ghosts

This was done by the procedure of Downes & Michell (1981). Blood (10 ml) obtained from a volunteer, with 5 mM-EDTA used as an anticoagulant, was centrifuged, and plasma and leucocytes were carefully removed by aspiration. The erythrocyte pellet was washed three times with 154 mM- NaCl /1.5 mM-Hepes, pH 7.2. The washed erythrocytes were incubated at 37°C for 2 h in a buffer containing 30 μCi of $[\text{^32P}]\text{P}_i$ /ml, 136.9 mM- NaCl , 2.7 mM- KCl , 1.8 mM- CaCl_2 , 1.0 mM- MgCl_2 , 11.9 mM- NaHCO_3 , 5.5 mM-glucose and 10 mM-Hepes, pH 7.4. Erythrocyte ghosts were isolated by lysis with 20 mM-Tris/1 mM-EDTA, pH 7.4, washed three times with 20 mM-Tris/1 mM-EDTA, pH 7.4, and suspended in 5 ml of 20 mM-Tris, pH 7.4, without EDTA. The suspended erythrocyte ghosts were incubated with 1 mM- Ca^{2+} at 37°C for 15 min, and the reaction was terminated by addition of 5 ml of 10% trichloroacetic acid. After centrifugation, the supernatant was stored at -20°C until use.

Preparation of ^3H -labelled $\text{Ins}(1,3,4)\text{P}_3$ from rat parotid gland

Rat parotid glands were removed, minced with scissors, and chopped with a McIlwain tissue chopper. After several washes with inositol-free DMEM/Hepes medium, phospholipids were labelled at 37°C for 90 min in 5 ml of inositol-free DMEM containing 8 μCi of $[\text{^3H}]$ inositol/ml. The slices were washed with EMEM/Hepes buffer and incubated for an additional 60 min at 37°C . The slices were washed again with EMEM/Hepes buffer and challenged with 1 mM-carbachol at 37°C for 15 min in the presence of 10 mM- LiCl . The reaction was terminated by addition of an equal volume of 10% trichloroacetic acid, the samples were centrifuged, and the supernatant fraction was kept for h.p.l.c. analysis.

Preparation of cytosol fractions from rat brain and 1321N1 cells

A whole rat brain was carefully removed, washed with 0.15 M-sucrose, and homogenized in 50 vol. of 0.15 M-sucrose with a Teflon/glass homogenizer. The homogenate was centrifuged at 45000 *g* for 15 min, and the supernatant was decanted and stored at -20°C . 1321N1 cells were washed three times with 0.15 M-sucrose, the surface was scraped, and the suspended cells were homogenized in 0.5 ml of 0.15 M-sucrose by using 20 strokes of a Teflon/glass homogenizer. The cytosol fraction was obtained by centrifugation at 15600 *g* for 5 min.

Determination of InsP_3 isomers by h.p.l.c.

InsP_3 isomers were analysed by the method of Irvine *et al.* (1985). The samples (approx. 1 ml) were washed four times with diethyl ether to remove trichloroacetic acid, and injected into a Waters h.p.l.c. system equipped with a Whatman Partisil SAX 10 anion-exchange column (4.6 mm $\times$ 250 mm) equilibrated with water and maintained at a flow rate of 1.25 ml/min. ATP, ADP and AMP (5 nmol of each) were used as u.v. markers. The injection of samples was followed by a 5 min flow of water and a 24 min flow of a linear gradient of 0–1.0 M-ammonium formate (pH 3.7) buffered by H_3PO_4 (AF/P buffer). After a 6 min flow (29–35 min of elution) of AF/P buffer (1.0 M), the eluent was changed to water and

elution was continued for the following 17 min (35–52 min of elution) before the next sample was injected. Eluates were collected at 1 min intervals for the first 30 min and at 0.3 min intervals after 30 min. A modified programme of elution was used when only InsP_3 isomers were measured. After injection of the sample, the concentration of AF/P buffer was increased to 0.71 M. After 6 min, the concentration of AF/P buffer was increased linearly to 1.0 M until 13 min, and elution with 1.0 M buffer maintained until 20 min. After that, the eluent was changed to water (20–22 min), and water was allowed to flow for 5 min (22–27 min) before the next sample was injected. When the modified elution pattern was used, the eluates were collected at 0.3 min intervals beginning 1 min before the elution of ATP. The retention times of inositol phosphates and adenine nucleotides gradually changed during repeated use of the h.p.l.c. columns. Therefore, the retention times of adenine nucleotides were checked each day before injection of radio-labelled inositol phosphates.

Determination of InsP_4 by h.p.l.c.

In experiments designed to detect the formation of InsP_4 , the linear gradient used for InsP_3 elution was extended to 1.8 M-ammonium formate buffered by H_3PO_4 (pH 3.7). After the sample was injected, water was passed through the column for 5 min, and then a linear gradient of 0–1.8 M-AF/P buffer was passed within 43 min (5–48 min of elution). Elution was maintained with 1.8 M-AF/P buffer for 5 min (48–53 min), and linearly changed to water (53–57 min). Water was passed through the column for an additional 8 min (57–65 min) before a new sample was injected. The eluate was collected at 0.3 min intervals for InsP_3 isomers and at 1 min intervals of InsP_4 . A modified programme of elution was used when both InsP_3 isomers and InsP_4 were measured. After injection of the sample, the concentration of AF/P buffer was increased to 0.7 M. After 6 min, the concentration of AF/P buffer was increased linearly to 1.8 M until 32 min, and the elution with 1.8 M buffer maintained until 37 min. The eluent then was changed to water.

RESULTS

As reported previously by Masters *et al.* (1985a), exposure of 1321N1 cells to carbachol results in a linear accumulation of inositol phosphates for 60 min (Fig. 1). In contrast, exposure of 1321N1 cells to histamine resulted in a marked increase in inositol phosphate accumulation during the first 1 min, but thereafter histamine produced only a small effect relative to carbachol (Fig. 1). Addition of fresh histamine to cells previously exposed to histamine for 15 min caused no further increase in inositol phosphate formation (Fig. 1, inset). In contrast, addition of carbachol to histamine-pretreated cultures resulted in inositol phosphate accumulation at a rate at least as rapid as that observed during incubation with carbachol alone (Fig. 1, inset).

Measurement of total inositol phosphate accumulation in the presence of LiCl obviously does not provide a complete analysis of the relative capacity of carbachol and histamine to stimulate phosphoinositide breakdown. Since carbachol and histamine initially stimulate Ca^{2+} mobilization and Ca^{2+} -calmodulin-sensitive cyclic AMP

phosphodiesterase to a similar extent in 1321N1 cells (Nakahata *et al.*, 1986), the relative effectiveness of these agonists for stimulation of accumulation of $\text{Ins}(1,4,5)\text{P}_3$ was compared.

The h.p.l.c. method of Irvine *et al.* (1985) was used to separate InsP_3 isomers. The elution pattern of inositol compounds isolated from carbachol-stimulated 1321N1 cells was similar to that previously reported for rat parotid gland (Irvine *et al.*, 1985). In the elution profile illustrated in Fig. 2, most of the radioactivity was in the form of four major radioactive peaks. On the basis of the similarity of the retention times of these radioactive species to those of the inositol compounds in the report by Irvine *et al.* (1985), as well as their positions relative to standards of AMP, ADP, and ATP, these peaks of radioactive compounds apparently consisted predominantly of free inositol (peak A), Gro-PIIns (peak B), InsP_1 (peak C), InsP_2 (peak D) and Gro-PIInsP_2 (peak E). In this experiment very little radioactivity was eluted with a retention time similar to that of ATP. However, expansion of the ordinate (inset, Fig. 2) illustrates that two peaks of radioactivity were eluted in this region. In the experiment illustrated in Fig. 3, these peaks are more

Fig. 1. Accumulation of total inositol phosphates in response to carbachol and histamine in 1321N1 cells

Cells were labelled overnight with 1 μCi of $[^3\text{H}]\text{inositol}/\text{ml}$, washed with EMEM/Hepes and incubated with 1 mM-carbachol (●), 1 mM-histamine (■) or vehicle (△) for the indicated times. Data are presented as the accumulation of total inositol phosphates with time, and are means $\pm$ S.E.M. for triplicate dishes. Similar results were obtained in three experiments. Inset: cells were incubated with 1 mM-histamine (○) or 1 mM-carbachol (△) for 15 min. Fresh histamine (1 mM; ●) or carbachol (1 mM; ▲) was then added to the histamine-pretreated cells, and total inositol phosphate accumulation (ordinate) measured at the indicated times. The data are the means of triplicate dishes; similar results were obtained in two separate experiments.

Fig. 2. H.p.l.c. elution profile of [^3H]inositol compounds

1321N1 cells were labelled overnight with $5\ \mu\text{Ci}$ of [^3H]inositol/ml. The cells were incubated with $1\ \text{mM}$ -carbachol for $1\ \text{min}$ in the absence of LiCl , and the incubation was terminated by addition of $0.5\ \text{ml}$ of 5% trichloroacetic acid. Trichloroacetic acid was removed by four washes with diethyl ether, and ether was removed by a stream of N_2 gas. Then $1\ \text{ml}$ of the sample was injected with ATP, ADP and AMP as described in the Materials and methods section. The retention time of each marker is indicated by the arrows. Inset: some of the same data are shown in an expanded scale. Peaks A, B, C, D, E, F and G correspond to inositol, Gro- P_{Ins} , $\text{Ins}P_1$, $\text{Ins}P_2$, Gro- $P_{\text{Ins}}P_2$, $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(1,4,5)P_3$ respectively.

prominent, and a comparison of their elution profile with that of inositol phosphates isolated from human erythrocytes and rat parotid is presented. It has been reported previously that $\text{Ins}(1,3,4)P_3$ is co-eluted with ATP (Irvine *et al.*, 1985). A peak of radioactivity from carbachol-treated 1321N1 cells is co-eluted with ATP, and with a concentration of ammonium formate and a retention time similar to that previously reported for $\text{Ins}(1,3,4)P_3$ (Irvine *et al.*, 1985). Since parotid glands stimulated with carbachol under the conditions described in the Materials and methods section predominantly contain $\text{Ins}(1,3,4)P_3$ (Irvine *et al.*, 1984), the data presented in Fig. 3(c) also support the tentative identity of the first radioactive species illustrated in this elution profile. That is, this compound(s) is co-eluted with the predominant $\text{Ins}P_3$ isomer from parotid. Irvine *et al.* (1985) reported that $\text{Ins}(1,4,5)P_3$ was eluted with a somewhat greater retention time than ATP and $\text{Ins}(1,3,4)P_3$. In addition, Downes & Michell (1981) have reported that stimulation of erythrocyte ghosts with Ca^{2+} produces large amounts of $\text{Ins}(1,4,5)P_3$. Thus the retention time of the second radioactive species in Fig. 3 and its coelution with $\text{Ins}P_3$ from human erythrocytes are consistent with its identity as $\text{Ins}(1,4,5)P_3$.

In the absence of carbachol or histamine, little if any radioactivity could be detected in 1321N1 cells as the species tentatively identified as $\text{Ins}(1,3,4)P_3$ (Fig. 4); a prominent peak of radioactivity was eluted as the species tentatively identified as $\text{Ins}(1,4,5)P_3$. Addition of carbachol or histamine resulted in a rapid increase in the amount of each species (Figs. 4 and 5). In contrast with the results obtained with rat parotid gland (Irvine *et al.*, 1985), both $\text{Ins}P_3$ isomers accumulated with similar

kinetics in the presence of each agonist. Maximal values were attained for each isomer with each agonist within $30\ \text{s}$. $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ contents remained elevated for at least $60\ \text{min}$ of exposure to carbachol (Fig. 5, and results not shown). In contrast, the amounts of both $\text{Ins}P_3$ isomers returned to control values within $5\ \text{min}$ of exposure to histamine. The data suggest that desensitization to the effects of histamine on $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ accumulation occurs rapidly, whereas muscarinic-receptor-mediated increases in $\text{Ins}P_3$ formation are maintained during prolonged exposure to agonists.

The disappearance of $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ also was examined in 1321N1 cells after cessation of hormone stimulation. As previously illustrated (Fig. 5), $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ were significantly increased during a $5\ \text{min}$ challenge with carbachol; contents rapidly declined after the addition of $10\ \mu\text{M}$ -atropine (Fig. 6). Although some variability was observed, in three out of four experiments the rate of disappearance of $\text{Ins}(1,3,4)P_3$ was more rapid than that of $\text{Ins}(1,4,5)P_3$.

On the basis of the proposal that $\text{Ins}(1,3,4)P_3$ may be derived from $\text{Ins}(1,3,4,5)P_4$ (Batty *et al.*, 1985; Irvine *et al.*, 1986), the potential presence of $\text{Ins}(1,3,4,5)P_4$ was examined in 1321N1 cells. Irvine *et al.* (1986) have demonstrated that [^3H] $\text{Ins}(1,4,5)P_3$ can be converted into [^3H] $\text{Ins}(1,3,4,5)P_4$ by an $\text{Ins}(1,4,5)P_3$ 3-kinase in rat brain cytosol. In support of the data of Irvine *et al.* (1986), incubation of [^3H] $\text{Ins}(1,4,5)P_3$ with a cytosol fraction obtained from rat brain resulted in conversion into a radioactive species exhibiting h.p.l.c. elution properties similar to those previously described for $\text{Ins}(1,3,4,5)P_4$ (Fig. 7). Incubation of [^3H] $\text{Ins}(1,4,5)P_3$ with cytosol

Fig. 3. Characterization of InsP_3 isomers in 1321N1 cells

H.p.l.c. elution of ^3H inositol phosphates was as described in the Materials and methods section. Data are presented for fractions collected at 0.3 min intervals, beginning 30 min after injection of samples. The retention time of standard ATP co-injected with sample A is indicated. The results are representative of those obtained in four such comparisons. (a) 1321N1 cells were labelled overnight with $10\ \mu\text{Ci}$ of ^3H inositol/ml. The cells were incubated with carbachol (1 mM) for 1 min in the absence of LiCl. (b) A ^3H inositol phosphate sample from carbachol-stimulated 1321N1 cells was combined with an inositol [^{32}P]phosphate sample prepared from human erythrocyte ghosts as described in the Materials and methods section. The combined sample was injected and h.p.l.c. elution carried out as described in the Materials and methods section. (c) A ^3H inositol phosphate fraction was prepared from rat parotid and subjected to h.p.l.c. ion-exchange chromatography as described in the Materials and methods section.

obtained from 1321N1 cells also resulted in appearance of a radioactive species exhibiting the same retention time (Fig. 7c). Moreover, this radioactive species was observed in intact 1321N1 cells after incubation with carbachol for 1 min (Fig. 7d). On the basis of its

Fig. 4. Time-dependent accumulation of InsP_3 isomers in carbachol-stimulated 1321N1 cells

Cells were labelled overnight with $10\ \mu\text{Ci}$ of ^3H inositol/ml. The cells were incubated with 1 mM-carbachol for 5 s to 5 min in the absence of LiCl. Incubation was terminated by addition of 5% trichloroacetic acid, and h.p.l.c. elution of ^3H inositol phosphates was carried out as described in the Materials and methods section. Data are presented for fractions collected at 0.3 min intervals, beginning 18 min after injection of samples. The results are representative of those obtained in four experiments. Arrows in the top left panel indicate positions of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$.

co-elution during h.p.l.c. ion-exchange chromatography with the radioactive species produced by incubation of ^3H $\text{Ins}(1,4,5)\text{P}_3$ with rat brain cytosol, we have tentatively identified this product as $\text{Ins}(1,3,4,5)\text{P}_4$. Its identity as $\text{Ins}(1,3,4,5)\text{P}_4$ is further supported by the observation that, during extended incubation of 1321N1 cell or brain homogenate with ^3H $\text{Ins}(1,4,5)\text{P}_3$, a radioactive species exhibiting the elution properties of $\text{Ins}(1,3,4)\text{P}_3$ was formed (results not shown). As illustrated in Fig. 8, the compound identified as $\text{Ins}(1,3,4,5)\text{P}_4$ was present in non-stimulated 1321N1 cells, and its concentration was increased by approx. 3-fold in the presence of 1 mM-carbachol. The time course of accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$ was essentially identical with that of $\text{Ins}(1,4,5)\text{P}_3$ measured in the same cells (Fig. 8). Atropine blocked the effects of carbachol on the accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$ (results not shown). Histamine stimulated a 2–3-fold increase in $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation that was maximal within 30 s and that, in contrast with the effects of carbachol, returned to control values within 5 min (results not shown).

Fig. 5. Time course of accumulation of InsP_3 isomers in the presence of carbachol and histamine

1321N1 cells were labelled overnight with $10 \mu\text{Ci}$ of $[^3\text{H}]\text{inositol}/\text{ml}$. The cells were incubated for the indicated time in the absence of LiCl , and the reaction was terminated by addition of 0.5 ml of 5% trichloroacetic acid. InsP_3 isomers were separated by h.p.l.c. by the modified elution protocol described in the Materials and methods section. $\text{Ins}(1,4,5)\text{P}_3$ (●) and $\text{Ins}(1,3,4)\text{P}_3$ (▲) were quantified by pooling the fractions corresponding to each InsP_3 isomer. Amounts of $\text{Ins}(1,4,5)\text{P}_3$ (○) and $\text{Ins}(1,3,4)\text{P}_3$ (△) in the absence of added drug are also indicated. Each point represents the mean $\pm$ S.E.M. for three or four dishes; the results are similar to those obtained in three separate experiments.

DISCUSSION

The kinetics of accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in 1321N1 cells in response to both histamine and carbachol are consistent with the proposed role of this inositol phosphate as the receptor-regulated second messenger involved in mobilization of intracellular Ca^{2+} . Intracellular Ca^{2+} concentration measured with Quin2 in intact 1321N1 cells (J. R. Hepler & T. K. Harden, unpublished work) increased with a time course similar to that for $\text{Ins}(1,4,5)\text{P}_3$, and, as with $\text{Ins}(1,4,5)\text{P}_3$, Ca^{2+} concentrations reached a maximum within approx. 30 s. In contrast, the kinetics of accumulation of $\text{Ins}(1,3,4)\text{P}_3$ in 1321N1 cells in response to carbachol and histamine

Fig. 6. InsP_3 contents after addition of atropine to carbachol-stimulated 1321N1 cells

Cells were labelled overnight with $10 \mu\text{Ci}$ of $[^3\text{H}]\text{inositol}/\text{ml}$. The cells were incubated for 5 min with 1 mM -carbachol. Atropine ($10 \mu\text{M}$) was then added to the medium and incubation continued for the indicated times. The amounts of $\text{Ins}(1,4,5)\text{P}_3$ (○, ●) and $\text{Ins}(1,3,4)\text{P}_3$ (△, ▲) were determined by h.p.l.c. analysis of samples taken before (○, △) or after (●, ▲) the addition of atropine. The data are means $\pm$ S.E.M. for three dishes, and are representative of results obtained in three separate experiments.

were surprising. That is, $\text{Ins}(1,3,4)\text{P}_3$ accumulated with a time course essentially the same as that of $\text{Ins}(1,4,5)\text{P}_3$. Furthermore, on addition of atropine, $\text{Ins}(1,3,4)\text{P}_3$ disappeared as fast as, if not faster than, $\text{Ins}(1,4,5)\text{P}_3$. Although the physiological role of $\text{Ins}(1,3,4)\text{P}_3$ is not known, its relatively slow rate of appearance and disappearance in other target tissues has not been compellingly appropriate for a role of this inositol phosphate in hormone-stimulated Ca^{2+} mobilization. This point cannot be made for 1321N1 cells, where the time courses of accumulation and disappearance of $\text{Ins}(1,3,4)\text{P}_3$ are very similar to those of $\text{Ins}(1,4,5)\text{P}_3$ and intracellular Ca^{2+} . It also should be emphasized that the fold stimulation of $\text{Ins}(1,3,4)\text{P}_3$ accumulation in the presence of carbachol was much greater than for $\text{Ins}(1,4,5)\text{P}_3$.

Batty *et al.* (1985) have reported that stimulation of rat cortical slices with carbachol results in the rapid formation of the novel inositol phosphate, $\text{Ins}(1,3,4,5)\text{P}_4$. This could be formed either by the hydrolysis of phosphatidylinositol 3,4,5-trisphosphate or by the phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$. The latter mechanism appears more likely, since incubation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ with cytosol from rat brain, liver or pancreas, as well as micro-injection of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ into *Xenopus* oocytes,

Fig. 7. H.p.l.c. elution of $\text{Ins}(1,3,4,5)\text{P}_4$

$[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (50 pmol; 250 nM) was incubated with rat brain cytosol for 30 s (b), with cytosol from 1321N1 cells for 5 min (c), or without cytosol for 5 min (a). Incubations were in the presence of 10 mM-ATP, 20 mM- MgCl_2 and 100 mM-Hepes (pH 7.5) in a final volume of 0.2 ml at 37 °C. Intact 1321N1 cells (d) prelabelled with $[\text{H}]\text{inositol}$ were incubated with carbachol (1 mM) for 1 min. The reactions were terminated as described in the Materials and methods section and the samples were applied to h.p.l.c. A modified programme of elution for InsP_3 and InsP_4 as described in the Materials and methods section was used. The data are representative of results obtained in three experiments.

all resulted in the formation of InsP_4 (Irvine *et al.*, 1986). Preliminary characterization of the $\text{Ins}(1,4,5)\text{P}_3$ kinase involved also has been reported (Irvine *et al.*, 1986). The data of Batty *et al.* (1985) suggest that $\text{Ins}(1,3,4,5)\text{P}_4$ is the likely source of $\text{Ins}(1,3,4)\text{P}_3$ through the action of a 5-phosphatase. On the basis of properties of elution from h.p.l.c. and enzymic conversion of exogenous $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$, we have tentatively identified a radioactive species from carbachol-stimulated 1321N1 cells as $\text{Ins}(1,3,4,5)\text{P}_4$. Its kinetics of appearance and disappearance during hormonal challenge of 1321N1 cells are remarkably similar to those of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$. None of the work in this manuscript directly addressed the source of these inositol phosphates. However, if the precursor-product relationship of $\text{Ins}(1,4,5)\text{P}_3 \rightarrow \text{Ins}(1,3,4,5)\text{P}_4 \rightarrow \text{Ins}(1,3,4)\text{P}_3$ applies in hormone-stimulated 1321N1 cells, then the kinase and

Fig. 8. Time course of accumulation of $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ in carbachol-stimulated 1321N1 cells

Cells were labelled overnight with 10 μCi of $[\text{H}]\text{inositol}/\text{ml}$. The cells were incubated with carbachol (1 mM) in the absence of LiCl, and the reaction was terminated by 0.5 ml of 5% trichloroacetic acid. InsP_3 isomers and InsP_4 were separated by h.p.l.c. as described in the Materials and methods section. Data are means $\pm$ S.E.M. for three dishes; similar results were obtained in two separate experiments. $\text{Ins}(1,4,5)\text{P}_3$ ($\circ$, $\bullet$), $\text{Ins}(1,3,4)\text{P}_3$ ($\triangle$, $\blacktriangle$) and $\text{Ins}(1,3,4,5)\text{P}_4$ ($\square$, $\blacksquare$) accumulations in the absence ($\circ$, $\triangle$, $\square$) or presence ($\bullet$, $\blacktriangle$, $\blacksquare$) of carbachol are shown.

phosphatase involved must be very active. It is important to note that in preliminary experiments with washed membranes from 1321N1 cells (N. Nakahata & T. K. Harden, unpublished work), carbachol in the presence of guanosine 5'-[γ -thio]triphosphate stimulated the formation of $\text{Ins}(1,4,5)\text{P}_3$, but not $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,3,4)\text{P}_3$. One explanation for this result would be the loss in a membrane preparation of the contribution of a soluble kinase that converts $\text{Ins}(1,4,5)\text{P}_3$ into $\text{Ins}(1,3,4,5)\text{P}_4$. Two other radioactive species from 1321N1 cells are also eluted at very high ammonium formate molarities during h.p.l.c. ion-exchange chromatography (N. Nakahata & T. K. Harden, unpublished work). The elution properties of these species are similar to that expected for InsP_5 and InsP_6 (Heslop *et al.*, 1985); however, stimulation of 1321N1 cells with carbachol or histamine has no effect on the amounts of these two compounds.

Although both muscarinic- and histamine-receptor activation results in inositol phosphate formation in 1321N1 cells, the properties of these responses are markedly different. The initial rates of carbachol- or histamine-stimulated accumulation, measured either as total inositol phosphates or as $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ accumulation, were similar. However, the accumulation of InsP_3 isomers in the presence of carbachol reached a steady state that was maintained for

up to 60 min, whereas both InsP_3 isomers returned to control values within 5 min of exposure to histamine. Changes in the amounts of total inositol phosphates in response to the two agonists reflected these marked differences in the production of InsP_3 . Since re-addition of histamine after 5–10 min had no further effect on inositol phosphate formation, the results indicate that the response of 1321N1 cells to histamine is rapidly desensitized, whereas, as has been previously demonstrated by Masters *et al.* (1985a), the muscarinic-receptor-mediated response does not. Although agonist-occupied H_1 -histamine (Nakahata *et al.*, 1986) and muscarinic (Hughes *et al.*, 1984; Evans *et al.*, 1985; Martin *et al.*, 1985; Masters *et al.*, 1985b) receptors apparently interact with the same population of G-protein, which is coupled to phospholipase C and is not a pertussis-toxin substrate, preincubation of 1321N1 cells with histamine does not decrease the subsequent capacity of carbachol to stimulate the accumulation of inositol phosphates. Thus the simplest interpretation of the results is that histamine responses are selectively modified at the level of the histamine receptor or at the level of interaction of this receptor with the putative guanine nucleotide regulatory protein involved in coupling hormone receptors to phospholipase C. No evidence for agonist-induced functional modification of the muscarinic receptor, other than receptor down-regulation after long-term exposure to carbachol, has been detected in 1321N1 cells (Masters *et al.*, 1985a; Harden *et al.*, 1985).

Although homologous desensitization of the histamine receptor response apparently occurs at the level of the plasma membrane, and the effects of muscarinic-receptor stimulation on inositol phosphate formation do not decrease for up to 60 min, responsiveness measured at subsequent steps rapidly declines for both agonists in a heterologous manner (Masters *et al.*, 1985a; Harden *et al.*, 1986). That is, the Ca^{2+} -mobilizing response as well as the response to carbachol and histamine of Ca^{2+} -calmodulin-regulated cyclic AMP phosphodiesterase are rapidly lost after treatment with carbachol. Depletion of a hormone-sensitive Ca^{2+} pool or desensitization of InsP_3 'receptors' could account for heterologous desensitization occurring at a step after inositol phosphate formation.

In conclusion, carbachol as well as histamine stimulate the formation of compounds tentatively identified as $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$. Since the rates of appearance and disappearance of all three compounds are very similar in 1321N1 cells, the kinase and phosphatase involved must be very active if the precursor-product relationship of $\text{Ins}(1,4,5)\text{P}_3 \rightarrow \text{Ins}(1,3,4,5)\text{P}_4 \rightarrow \text{Ins}(1,3,4)\text{P}_3$ applies. H_1 -Histamine-receptor-mediated, but not muscarinic-receptor-mediated, inositol phosphate formation is rapidly desensitized in 1321N1 cells. Application of

newly developed methodology for the analysis of phosphoinositide turnover in cell-free preparations from 1321N1 cells (Hepler & Harden, 1986) should allow identification of the level at which the regulation of the muscarinic- and histamine-receptor responses differ.

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