Histamine- H_1 -receptor-mediated phosphoinositide hydrolysis, Ca^{2+} signalling and membrane-potential oscillations in human HeLa carcinoma cells

Ben C. TILLY,*† Leon G. J. TERTOOLEN,* A. Caro LAMBRECHTS,* Rolf REMORIE,* Siegfried W. DE LAAT* and Wouter H. MOOLENAAR†

*Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, and †The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

In human HeLa carcinoma cells, histamine causes a dose-dependent formation of inositol phosphates, production of diacylglycerol and a transient rise in intracellular $[Ca^{2+}]$. These responses are completely blocked by the H_1 -receptor antagonist pyrilamine. In streptolysin-O-permeabilized cells, formation of inositol phosphates by histamine is strongly potentiated by guanosine 5'-[γ -thio]triphosphate and inhibited by guanosine 5'-[β -thio]diphosphate, suggesting the involvement of a GTP-binding protein. Histamine stimulates the rapid but transient formation of $Ins(1,4,5)P_3$, $Ins(1,3,4)P_3$ and $InsP_4$. $InsP$ accumulates in a much more persistent manner, lasting for at least 30 min. Studies with streptolysin-O-permeabilized cells indicate that $InsP$ accumulation results from dephosphorylation of $Ins(1,4,5)P_3$, rather than direct hydrolysis of $PtdIns$. The rise in intracellular $[Ca^{2+}]$ is biphasic, with a very fast release of Ca^{2+} from intracellular stores, that parallels the $Ins(1,4,5)P_3$ time course, followed by a more prolonged phase of Ca^{2+} influx. In individual cells, histamine causes a rapid initial hyperpolarization of the plasma membrane, which can be mimicked by microinjected $Ins(1,4,5)P_3$. Histamine-induced hyperpolarization is followed by long-lasting oscillations in membrane potential, apparently owing to periodic activation of Ca^{2+} -dependent K^+ channels. These membrane-potential oscillations can be mimicked by microinjection of guanosine 5'-[γ -thio]triphosphate, but are not observed after microinjection of $Ins(1,4,5)P_3$. We conclude that H_1 -receptors in HeLa cells activate a $PtdInsP_2$ -specific phospholipase C through participation of a specific G-protein, resulting in long-lasting oscillations of cytoplasmic free Ca^{2+} .

INTRODUCTION

There is ample evidence that histamine is involved in a number of important physiological responses, such as neurotransmission, inflammation, allergic reactions and gastric acid secretion [1,2]. Histamine has also been suggested to be involved in certain types of cell proliferation, such as wound healing, embryonic development and tumour growth [3]. Histamine receptors are classified into three subtypes, H_1 -, H_2 - and H_3 -receptors, which differ in their sensitivity to antagonists and mediate different actions [4–7]. Whereas H_1 - and H_2 -receptors are widely distributed throughout the body, the H_3 -subtype has been reported to occur in nervous tissue and mast cells only [5,6,8]. H_1 -receptor activation leads to a rapid breakdown of inositol lipids [9–11], whereas H_2 -receptors are coupled to the cyclic-AMP-generating system [12].

We have recently obtained evidence that histamine, acting through H_1 -receptors, can function as a growth factor and chemo-attractant for human HeLa carcinoma cells [12a]. However, the nature of the mitogenic and chemotactic signals generated by the H_1 -type receptor in HeLa cells is not yet fully understood. In the present

study, we have analysed the effects of histamine on phosphoinositide hydrolysis, Ca^{2+} signalling and diacylglycerol formation, using both intact and permeabilized HeLa cells. Furthermore, we show that during histaminergic activation of phospholipase C marked oscillations in membrane potential occur within individual cells, that can be mimicked by microinjection of stable GTP analogues. Such long-lasting oscillations, which are attributable to repetitive increases in $[Ca^{2+}]_i$, may have a signalling role in H_1 -receptor-mediated chemotaxis and cell proliferation.

MATERIALS AND METHODS

Materials

Cimetidine, DiC_8 , histamine, pyrilamine, PMA and 4 α PDD were purchased from Sigma. Other agents were obtained from the following sources: fetal-calf serum from Hyclone, guanine nucleotides from Boehringer Mannheim, indo-1 acetoxymethyl ester from Molecular Probes, and streptolysin-O from Wellcome Nederland B.V. *myo*-[2- 3H]Inositol (12.3 Ci/mmol) and [2- 3H]-

Abbreviations used: 4 α PDD, 4 α -phorbol 12,13-didecanoate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DiC_8 , 1,2-dioctanoyl-*rac*-glycerol; DMEM, Dulbecco's Modified Eagle's Medium; GTP[S], guanosine 5'-[γ -thio]triphosphate; $InsP_n$, fraction containing $InsP$, $InsP_2$ and $InsP_3$; PMA, phorbol 12-myristate 13-acetate.

† To whom reprint requests should be addressed. Present address: Department of Biochemistry I, Medical Faculty, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

glycerol (1 Ci/mmol) were purchased from Amersham International.

Cell culture

Human HeLa carcinoma cells were routinely grown in DMEM containing 7.5% (v/v) fetal-calf serum. The cells were grown to near-confluency before being used in the experiments.

Determination of total inositol phosphates

Cells were prelabelled to near-equilibrium in serum-free DMEM/Ham's F-12 (1:1, v/v) medium containing 10 µg of transferrin/ml and 2 µCi of *myo*-[2-³H]inositol for 24 h. At 2 h before stimulation, the cultures were shifted to serum-free DMEM containing 10 mM-Hepes (pH 7.4). The cells were stimulated with histamine for various periods in the presence of 10 mM-LiCl, and incubations were terminated by replacing the medium by 10% (v/v) trichloroacetic acid. After 10 min, extracts were collected and trichloroacetic acid was removed by washing with diethyl ether. The extracts were neutralized with Tris base and processed for analysis of the total [³H]inositol phosphate-containing fraction (InsP_n fraction) by anion-exchange chromatography on AG1 X8 columns (formate form; Bio-Rad) as previously described [13].

Separation of inositol phosphate by h.p.l.c.

A fraction containing inositol phosphates was prepared as described previously [13] and separated on a Partisil SAX column (250 mm × 4.6 mm; Whatman) at a flow rate of 1.25 ml/min. The gradient used was a modification of the methods described by Jackson *et al.* [14] and Hansen *et al.* [15]: distilled water (10 min), linear gradient to 0.5 M-ammonium formate/H₃PO₄ (pH 3.7; 12 min), convex gradient to 1.0 M (10 min; Waters Automatic Gradient Controller, gradient no. 2), followed by a linear gradient to 3.5 M (20 min). For quantification of the various inositol phosphates, 0.5 min (625 µl) fractions were collected and ³H radioactivity was determined.

Stimulation of permeabilized HeLa cells

Nearly confluent monolayers were permeabilized with streptolysin-O, essentially as described by Howell & Gomperts [16]. Briefly, cultures were incubated for 5 min in a buffer (137 mM-NaCl, 2.7 mM-KCl, 2 mM-EGTA, 1 mM-CaCl₂, 10 mM-LiCl, 20 mM-Pipes, 1 mg of bovine serum albumin/ml, 5.6 mM-glucose, 1 mM-ATP, pH 6.8) containing 0.4 i.u. of streptolysin-O. After washing the cells twice with buffer (1 ml), hormone and guanine nucleotides were added. The incubations were terminated by adding trichloroacetic acid (1 ml; 10%), and an inositol phosphate-containing fraction was prepared and analysed by both AG1 X8 or h.p.l.c. anion-exchange chromatography as described above.

Diacylglycerol production

Nearly confluent monolayers, grown on glass coverslips, were prelabelled with 10 µCi of [2-³H]glycerol/ml for 24 h and stimulated with histamine for various periods. Reactions were terminated by immersing the coverslips in chloroform/methanol (2:1, v/v) and lipids were extracted as described in [17]. Diacylglycerol was separated by high-performance t.l.c. on silica gel 60 (Merck) developed twice with hexane/diethyl ether/methanol/acetic acid (90:40:3:2, by vol.). Diacyl-

glycerol-containing spots were scraped, hydrolysed for 12 h with 0.6 M-HCl/methanol (3:2, v/v), and ³H radioactivity was determined.

Measurements of [Ca²⁺]_i

Nearly confluent HeLa monolayers, attached to rectangular coverslips, were incubated in serum-free DMEM/Ham's F-12 (1:1) medium containing 10 µg of transferrin/ml for 18 h. The cells were loaded with indo-1 by exposing them to 2 µM indo-1 ester for 40 min at 37 °C. [Ca²⁺]_i-dependent fluorescence was recorded and calibrated as described [18] at an excitation wavelength of 355 nm and an emission wavelength of 405 nm [19].

Electrophysiology

HeLa cells were fused by using poly(ethylene glycol) as described in [20]. This treatment results in the formation of small syncytia of 5–7 fused cells, which serve as a suitable model system for single-cell studies. Before being used in the experiments, the cells were allowed to recover for 2 h in DMEM containing 7.5% fetal-calf serum. A fine-tipped micro-pipette filled with 1 mM-GTP[S] was used for electrophoretic injections. Membrane potential and resistance were monitored with a second micro-electrode (3 M-KCl-filled; resistance: 30–50 MΩ). For further details see Tertoolen *et al.* [21]. Local extracellular application of histamine was done with a broken-tipped microelectrode filled with a hormone-containing solution (100 µM).

RESULTS

Histamine-induced inositol phosphate accumulation in HeLa cells

We have tested the effects of various hormones and mitogens for their ability to activate phospholipase C. As shown in Table 1, histamine (100 µM), bradykinin (1 µM) and bombesin (1 µM) were found to evoke an accumulation of inositol phosphates in the presence of Li⁺, indicative of phospholipase C-mediated breakdown of phosphoinositides. Among them, histamine is by far the most potent stimulus, giving an approx. 4-fold increase in InsP_n levels. Epidermal growth factor, although capable of raising [Ca²⁺]_i and inducing InsP_n formation in some other cell systems, including human carcinoma cells [22–24], has no significant effect on inositol phosphate formation, whereas fetal-calf serum (7.5%, v/v) raises inositol phosphate levels approx. 2-fold (Table 1).

The dose-dependence of the histamine-induced inositol phosphate formation was determined by stimulating Li⁺-pretreated cultures with different concentrations of hormone for 30 min. Fig. 1 shows that half-maximal stimulation occurs at approx. 1 µM, and saturation is observed at 10 µM. The histamine-induced inositol phosphate formation is blocked by low concentrations of the H₁-receptor antagonist pyrilamine: at 15 nM, the histamine response (100 µM) is decreased by 50% (results not shown). Inhibition by pyrilamine can be overcome by increasing the histamine concentration, as expected for a competitive inhibitor (results not shown).

Activation of phospholipase C by GTP[S] and AlF₄⁻

Using the streptolysin-O permeabilization protocol, we explored the possible involvement of a GTP-binding protein in the histamine-induced activation of phospho-

Table 1. Inositol phosphate formation by various Ca^{2+} -mobilizing hormones

[^3H]Inositol-prelabelled HeLa cells were incubated with the various hormones for 30 min in the presence of 10 mM-LiCl. An inositol-phosphates-containing fraction was prepared and analysed as described in the Materials and Methods section. Data are expressed as means $\pm$ S.E.M. for three determinations.

Stimulus	[^3H]InsP _n (d.p.m.)
None	1381 $\pm$ 71
Histamine (100 μM)	5286 $\pm$ 190
Bradykinin (1 μM)	2048 $\pm$ 119
Bombesin (1 μM)	1857 $\pm$ 114
Epidermal growth factor (100 ng/ml)	1397 $\pm$ 95
Fetal-calf serum (7.5 %, v/v)	2548 $\pm$ 105

lipase C. The accumulation of inositol phosphates in permeabilized HeLa cells treated with 100 μM of various guanine nucleotides is summarized in Table 2. The non-hydrolysable GTP analogue GTP[S] is a potent activator of phospholipase C, whereas other guanine nucleotides, including GTP and guanosine 5'-[$\beta\gamma$ -imido]triphosphate, are hardly effective. We confirmed that in intact cells neither GTP[S] nor guanosine 5'-[$\beta\gamma$ -imido]triphosphate induces inositol phosphate formation (results not shown). GTP[S] activates the breakdown of inositol phospholipids in a dose-dependent manner (Fig. 2a). Half-maximal stimulation is observed at a concentration of about 3 μM , and the response saturates at 10–20 μM -GTP[S]. At these saturating concentrations, inositol phosphate levels are increased by approx. 3-fold.

In the absence of GTP[S], histamine (100 μM) induces a small (less than 2-fold) increase in inositol phosphate formation, which can be blocked completely by guanosine 5'-[β -thio]diphosphate (1 mM; results not shown), an inhibitor of G-protein action. However, the response

Table 2. Effects of guanine nucleotides

Streptolysin-O-permeabilized cultures were treated with guanine nucleotides (100 μM) for 5 min. Inositol phosphate formation was determined as described in the Materials and methods section and normalized to 100 % (control). Abbreviations: GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate.

	[^3H]Inositol phosphate accumulation (% stimulation relative to control)
Control	100 $\pm$ 2
GMP	88 $\pm$ 4
GDP	99 $\pm$ 7
GDP[S]	110 $\pm$ 3
GTP	105 $\pm$ 7
p[NH]ppG	115 $\pm$ 1
GTP[S]	410 $\pm$ 8

to histamine is strongly potentiated when GTP[S] is present. An approx. 10-fold increase in the histamine-induced InsP_n accumulation is observed in the presence of 10 μM -GTP[S] (Fig. 2a).

To investigate the kinetics of phospholipase C activation by histamine and/or GTP[S], incubations were terminated at various time points after agonist addition. As shown in Fig. 2b, very little inositol lipid hydrolysis occurs in unstimulated permeabilized cells. Addition of histamine (100 μM) or GTP[S] (10 μM) alone causes an increase in inositol phosphates that lasts for at least 30 min. When histamine is added together with GTP[S], the initial rate of inositol phosphate formation (as measured over the first 10 min) is dramatically increased. After 10 min there is an approx. 20-fold increase above basal levels (Fig. 2b). Fig. 2(b) also shows that after a few minutes the rate of InsP_n production by simultaneously added histamine and GTP[S] levels off, a phenomenon probably due to depletion of PtdInsP₂ pools as a result of impaired resynthesis.

Treatment of intact cells or plasma-membrane preparations with fluoride in the presence of aluminium ions causes activation of nucleotide-dependent regulatory proteins, such as G_i, G_s and transducin [25–28]. We have studied the effects of AlF_4^- by treating permeabilized cells with increasing concentrations of NaF in the presence of 10 μM - AlCl_3 . Fig. 3 shows the increase in inositol phosphate formation evoked by NaF. Maximal effects are observed at 10 mM-NaF. Fig. 3 also shows that the combination of GTP[S] (10 μM) and histamine (100 μM) is equally potent as 10 mM-NaF/10 μM - AlCl_3 alone in stimulating InsP_n formation.

Duration of histamine-induced inositol phosphate formation

It is often observed that receptor-mediated responses are rapidly attenuated during prolonged receptor activation, a phenomenon generally referred to as homologous desensitization. To determine whether histamine-induced phosphoinositide hydrolysis in HeLa cells undergoes desensitization, we measured InsP_n accumulation during 30 min Li^+ pulses at various times after histamine addition. As shown in Fig. 4, even after 90 min of H₁-receptor stimulation, addition of LiCl still

Fig. 1. Dose-dependence of the histamine-induced inositol phosphate formation

[^3H]Inositol-prelabelled cultures were incubated with the indicated concentrations of histamine for 30 min in the presence of LiCl (10 mM). InsP_n accumulation was quantitated as described in the Materials and methods section. Each data point represents the mean $\pm$ S.E.M. for triplicate cultures.

Fig. 2. (a) Dose-dependency of inositol phosphate accumulation in permeabilized cells and (b) time course of inositol phosphate formation in streptolysin-O-treated cells

(a) [^3H]Inositol-prelabelled cells were permeabilized as described in the Materials and methods section and treated with various concentrations of GTP[S] for 10 min in the absence (○) or presence (●) of histamine (100 μM). (b) Cultures were stimulated with GTP[S] (10 μM ; G), histamine (100 μM ; H) or GTP[S] + histamine (10 μM and 100 μM respectively; G+H) for the indicated times. Control (C) represents [^3H]inositol phosphate formation in the absence of any stimulus. Each data point represents the mean $\pm$ S.E.M. for triplicate incubations.

gives an approx. 8-fold increase in total inositol phosphates, i.e. an unattenuated response, as compared with control incubations. These results indicate that no significant H_1 -receptor desensitization occurs over this time period. The amount of InsP_n accumulated during the successive Li^+ pulses even suggests that histamine-induced InsP_n formation increases with time, reaching maximal formation after 60–90 min (Fig. 4).

Fig. 3. Inositol phosphate formation induced by AlF_4^-

Streptolysin-O-permeabilized cells were stimulated with the indicated concentrations of NaF in the presence of 10 μM - AlCl_3 for 10 min. A fraction containing [^3H]inositol phosphates was prepared and analysed as described in the Materials and methods section. Bars represent inositol phosphate formation in control (C), GTP[S]-stimulated (10 μM ; G) and GTP[S] + histamine-stimulated (10 μM and 100 μM respectively; GH) cultures. Data are expressed as means $\pm$ S.E.M. ($n = 3$).

H.p.l.c. analysis

The major inositol phosphates and their isomers formed in response to histamine were resolved by h.p.l.c. anion-exchange chromatography in the absence of Li^+ . The time course of the various inositol phosphates formed after histamine stimulation shows two distinct phases.

Fig. 4. Duration of the histamine-induced phospholipase C activity

[^3H]Inositol-prelabelled cells were stimulated with histamine (100 μM) at zero time. LiCl (10 mM) was added at 0, 30, 60 or 90 min after histamine addition, and inositol phosphates were allowed to accumulate for 30 min. Thereafter, the incubations were terminated by replacing the medium with 10% trichloroacetic acid, and an InsP_n containing fraction was prepared. Data (means $\pm$ S.E.M., $n = 3$) represent the amount of InsP_n accumulated during a 30 min incubation with Li^+ in the absence (□) or presence (▨) of histamine.

Fig. 5. H.p.l.c. analysis of the histamine-induced inositol phosphate formation

[³H]Inositol-prelabelled cultures were incubated for the indicated times with 100 μM -histamine in the absence of Li⁺. Analysis of the individual inositol phosphates by anion-exchange h.p.l.c. was performed as described in the Materials and methods section. All data are derived from one representative experiment ($n = 3$). (a) InsP (○) and InsP₂ (●); (b) Ins(1,4,5)P₃ (○), Ins(1,3,4)P₃ (▼) and InsP₄ (●).

The first phase starts with a rapid, approx. 2-fold, increase in Ins(1,4,5)P₃, reaching peak values within 4–7 s, followed by a second and smaller peak (Fig. 5b). The Ins(1,4,5)P₃ formed is rapidly metabolized either to Ins(1,3,4)P₃, via Ins(1,3,4,5)P₄ (Fig. 5b), or directly to InsP₂ and InsP (Fig. 5a, inset) via pathways that are presumably very similar to those first described by Batty *et al.* [29]. This initial phase is followed by a second one, in which only InsP levels are elevated. This second phase of InsP formation starts at approx. 2–3 min after histamine addition and continues for at least 2 h (cf. Fig. 4), reaching a 4-fold increase above basal levels. No significant changes in InsP₂ and InsP₄ were observed.

In streptolysin-O-permeabilized cells an almost linear increase in Ins(1,4,5)P₃ and InsP₂ is observed, starting immediately after histamine/GTP[S] addition and lasting for at least 10 min (Fig. 6). Accumulation of InsP₄ and Ins(1,3,4)P₃, however, is delayed and becomes detectable only after a lag period of approx. 2 min, when Ins(1,4,5)P₃

levels are already increased several-fold. In contrast with the massive formation of InsP in intact cells, no significant increase in InsP occurs in permeabilized cells, suggesting that the prolonged InsP accumulation observed in intact cells is the result of dephosphorylation (via InsP₂) of Ins(1,4,5)P₃ rather than direct breakdown of inositol lipids other than PtdIns(4,5)P₂. In support of this notion, we observed that addition of unlabelled Ins(1,4,5)P₃ (2 μM) causes a significant decrease in the histamine/GTP[S]-stimulated formation of [³H]InsP₂ (results not shown).

Diacylglycerol production

In addition to the release of inositol phosphates, receptor-mediated hydrolysis of inositol lipids results in production of 1,2-diacylglycerol. Fig. 7 shows the time course of histamine-induced diacylglycerol formation during the first 30 min in [³H]glycerol-prelabelled cells. Diacylglycerol starts accumulating almost immediately

Fig. 6. H.p.l.c. analysis of the histamine + GTP[S]-induced inositol phosphate formation in permeabilized cells

[³H]Inositol-labelled cultures were permeabilized and stimulated with GTP[S] (10 μM) and histamine (100 μM) for the indicated times. An inositol phosphate-containing fraction was prepared and analysed as described in the Materials and methods section. All data are derived from a single experiment repeated twice. (a) InsP (○) and InsP₂ (●); (b) Ins(1,4,5)P₃ (▲), Ins(1,3,4)P₃ (□) and InsP₄ (■).

Fig. 7. Diacylglycerol formation induced by histamine

[${}^3\text{H}$]Glycerol-prelabelled cultures were stimulated with histamine ($100 \mu\text{M}$) for the indicated times. [${}^3\text{H}$]Diacylglycerol was quantified as described in the Materials and methods section. All data are derived from one representative experiment ($n = 3$).

after histamine addition, reaching a 50% increase between 1 and 5 min. The response is transient, and returns to basal levels within 10 min. Since inositol lipid hydrolysis continues for at least 2 h, the absence of sustained increase in diacylglycerol must be due to its rapid metabolism, either through degradation to monoacylglycerol or through phosphorylation to phosphatidic acid or both.

Inhibition of inositol phosphate formation through protein kinase C activation

In many cell types, synthetic diacylglycerols and phorbol esters inhibit receptor-mediated hydrolysis of polyphosphoinositides [24,30–32]. Pre-treatment of intact human HeLa cells with PMA causes a dose-dependent inhibition of the histamine-induced formation of inositol

Fig. 8. Inhibition of the histamine-induced inositol phosphate formation by pretreatment with PMA

[${}^3\text{H}$]Inositol-prelabelled cultures were treated with various concentrations of PMA for 15 min before stimulation with histamine ($100 \mu\text{M}$; 30 min). All incubations were done in the presence of 10 mM-LiCl. InsP_n accumulation in the absence of histamine and PMA is indicated ('Basal'). Data points represent means $\pm$ S.E.M. for triplicate cultures.

Table 3. Effects of protein kinase C activators on histamine-induced inositol phosphate formation

[${}^3\text{H}$]Inositol-labelled cultures were preincubated with PMA, DiC_8 and $4\alpha\text{PDD}$ (concentrations used: 100 ng/ml, $50 \mu\text{M}$ and 100 ng/ml respectively) for 15 min before histamine ($100 \mu\text{M}$; 30 min) addition. Down-regulation of protein kinase C was achieved by treating the cells with PMA (100 ng/ml) for 18 h. Inositol phosphate formation was quantified as described in the Materials and methods section.

Addition	[${}^3\text{H}$]Inositol phosphates (d.p.m.)	
	Control	Histamine
None	6897 ± 126	32960 ± 270
PMA (15 min)	7870 ± 587	8929 ± 135
(18 h)	8730 ± 243	37263 ± 1686
DiC_8	8756 ± 440	12346 ± 478
$4\alpha\text{PDD}$	6946 ± 234	31113 ± 907

phosphates (Fig. 8). Half-maximal inhibition was observed at a PMA concentration as low as 0.4 ng/ml. The effects of PMA can be mimicked by treating the cells with DiC_8 , a permeant synthetic diacylglycerol, but not by the inactive phorbol ester $4\alpha\text{PDD}$ (Table 3). In permeabilized cells, both the GTP[S] and histamine/GTP[S] responses were blocked after pre-treatment with TPA, suggesting protein kinase C-mediated feedback inhibition at or distal from the putative G-protein. Prolonged treatment of the cells with TPA (100 ng/ml; 18 h), in an attempt to down-regulate protein kinase C, does not significantly affect the magnitude of the histamine response (Table 3).

Rise in $[\text{Ca}^{2+}]_i$

Addition of histamine to HeLa cells loaded with the fluorescent Ca^{2+} indicator indo-1 results in a marked, transient, rise in $[\text{Ca}^{2+}]_i$ (Fig. 9). The histamine-induced Ca^{2+} signal consists of two distinct phases: an immediate and transient phase (reaching $1 \mu\text{M}$ free calcium within seconds) is followed by a smaller, but more prolonged, second phase, that lasts for approx. 4–5 min. The time course of the first phase closely resembles the kinetics of $\text{Ins}(1,4,5)\text{P}_3$ formation and is independent of extracellular calcium. When Ca^{2+} influx is blocked by excess EGTA or the Ca^{2+} -entry blocker La^{3+} , the second peak is entirely absent (Fig. 9b and 9c). Under these conditions, the remaining Ca^{2+} transient reflects the mobilization of Ca^{2+} from intracellular stores. As expected, brief pre-treatment of the cells with either pyrilamine (50 nM) or PMA (50 ng/ml) abolishes the Ca^{2+} signal completely (results not shown).

Oscillatory activation of Ca^{2+} -dependent K^+ channels

To investigate the Ca^{2+} response to histamine of HeLa cells in further detail, we used electrophysiological methods to monitor the behaviour of Ca^{2+} -activated K^+ channels in the plasma membrane. The opening of this voltage-insensitive channel is controlled by cytoplasmic free Ca^{2+} concentrations (range 0.1– $1 \mu\text{M}$) and results in membrane hyperpolarization [33]. The opening of Ca^{2+} -activated K^+ channels was monitored in single-cell preparations by direct intracellular recording. Brief appli-

Fig. 9. Histamine-induced rise in $[Ca^{2+}]_i$

Indo-1-loaded cells were stimulated with $100 \mu M$ -histamine (arrow) in the presence (a) or absence (b) of external Ca^{2+} . Ca^{2+} was removed by adding excess EGTA ($4 mM$) before histamine addition. (c) Histamine-induced $[Ca^{2+}]_i$ response in the presence of $100 \mu M$ - $La(NO_3)_3$.

Fig. 10. Membrane potential and resistance changes in response to local application of histamine

(a) Typical electro-physiological recording (negative upward) of the biphasic hyperpolarization after a short and local extracellular addition of histamine (HIST). (b) and (c) Effects of subsequent additions of $La(NO_3)_3$ (final concns. $100 \mu M$ and $200 \mu M$ respectively). Membrane resistance was monitored by the voltage response to hyperpolarizing current injections ($0.5 nA$). Resting potential: $-59 mV$.

cation of histamine (using a broken-tipped micropipette) to a single HeLa cell causes a transient, biphasic, hyperpolarization of the plasma membrane, accompanied by a small but statistically significant increase in membrane conductance (i.e. decrease in resistance; Fig. 10). Such hyperpolarizations have previously been described by Hazawa *et al.* [34], who showed that they are attributable to Ca^{2+} -activated K^+ channels. The observed change in membrane potential closely parallels the rise in $[Ca^{2+}]_i$ as measured in dye-loaded cell preparations (cf. Fig. 9). The initial hyperpolarizing phase is independent of extracellular Ca^{2+} and is followed by a second one that can be blocked by progressively increasing concentrations of La^{3+} (Fig. 10). The initial phase is larger in the presence of La^{3+} ions (Figs. 10b and 10c). This phenomenon is most likely due to an increased membrane stability, as indicated by a larger membrane resistance.

When the histamine concentration is increased to $100 \mu M$ (bath application), marked hyperpolarizing oscillations in membrane potential are recorded (Fig. 11). In several experiments these oscillations occurred at a frequency of 1.5 – $2.0 min^{-1}$ and lasted for at least 30 min in the continuous presence of histamine. These oscillations, which are most likely due to periodic changes in $[Ca^{2+}]_i$, last as long as histamine is present and cease immediately after removal of the hormone or addition of pyrilamine ($10 nM$; results not shown). Also, application of a dose of PMA ($25 ng/ml$) that completely inhibits histamine-induced phospholipase C activation, results in an immediate block of the observed membrane-potential responses (results not shown). Note that each hyperpolarization is followed by a substantial depolarization (approx. $10 mV$) and an increase in membrane resistance, suggesting the closure of certain ionic channels (Fig. 11). The ionic basis of this depolarization was not further investigated.

Fig. 11. Oscillations in membrane potential and resistance by histamine and GTP[S]

(a) Electrophysiological recording of membrane potential and resistance changes in response to a saturating concentration of histamine (HIST; 100 μ M). Membrane potential: -38 mV. (b) Membrane potential and resistance changes after iontophoretic microinjection of Ins(1,4,5)P₃ and GTP[S]. Injection current: 3.75 nA for 10 s. Membrane potential: -52 mV. Other details were as in Fig. 10.

Microinjected GTP[S] evokes oscillations

To gain insight into the possible mechanism(s) underlying the H₁-receptor-mediated oscillations, we microinjected Ins(1,4,5)P₃ and GTP[S]. Iontophoretic injection of pure Ins(1,4,5)P₃ into single cells evokes a marked transient hyperpolarization, owing to Ins(1,4,5)P₃-induced release of intracellularly stored Ca²⁺, without any sign of subsequent hyperpolarizing phases or oscillations (Fig. 11). In marked contrast, microinjection of GTP[S] evokes pronounced oscillatory changes in membrane potential and conductance, resembling those induced by histamine in both amplitude and frequency (about 1–2 min⁻¹; Fig. 11). GTP[S]-induced transients are, however, less sudden and more sinusoidal as compared with histamine-induced transients. Interestingly, these long-lasting oscillations in response to microinjected GTP[S] are immediately blocked after addition of PMA (100 ng/ml; results not shown).

DISCUSSION

Stimulation of cells types with certain hormones and/or growth factors causes a rapid activation of phospholipase C, resulting in the formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ and 1,2-diacylglycerol. In this report we have shown that histamine, a growth factor and a chemoattractant for human HeLa cells [35], evokes a sustained activation of phospholipase C in these cells,

leading to formation of various inositol phosphates and periodic changes in [Ca²⁺]_i.

Several lines of evidence indicate that a putative G-protein is involved in coupling the histamine H₁-receptor to phospholipase C. First, addition of both histamine and GTP[S] synergistically stimulates the formation of inositol phosphates. Second, although histamine itself does induce some inositol lipid hydrolysis (probably by virtue of endogenously produced GTP), guanosine 5'-[β -thio]diphosphate blocks this response completely. And finally, AlF₄⁻ has a strong stimulatory effect on inositol phosphate production. These results, along with the finding by others that GTP analogues shift the [³H]-mepyrrine-binding curve [36], strongly support the view that the H₁-receptor is coupled to a specific G-protein that mediates stimulation of phospholipase C.

Histamine-induced InsP_n formation in HeLa cells, as in other cell types [9–13], appears to be mediated by the pyrrolamine-sensitive H₁-type receptor. Our data show that H₁-receptor stimulation results in a rapid and sustained activation of phospholipase C, that lasts as long as the hormone is present. In contrast, treating cells with phorbol esters results in an immediate inhibition of inositol phosphate formation and Ca²⁺ release, suggesting that phospholipase C activation is under strong regulatory control by protein kinase C. Since levels of inositol-lipid-derived second messengers, in particular Ins(1,4,5)P₃ and 1,2-diacylglycerol, are only transiently elevated, presumably owing to their rapid metabolism, the lack of homologous desensitization could be explained by the absence of sustained diacylglycerol-mediated protein kinase C activation.

As a consequence of Ins(1,4,5)P₃ accumulation, a rise in [Ca²⁺]_i occurs almost immediately after histamine addition. The Ca²⁺ response can be separated into two phases, an initial phase which closely parallels the observed time course of Ins(1,4,5)P₃, followed by a second phase, representing La³⁺-sensitive Ca²⁺ influx. A correlation between InsP₄ formation and Ca²⁺ influx, as initially proposed by Irvine & Moore [37] and supported by the findings of others [38], is not readily observed from our data, although the InsP₄ peak, which is rather small in these cells, coincides with the time point of maximal Ca²⁺ influx.

Histamine induces a rapid membrane-potential hyperpolarization, apparently owing to an increase in the Ca²⁺-activated K⁺ conductance [33,34]. Although a brief local application of histamine results in a biphasic hyperpolarization, incubation of cells with saturating concentrations of histamine (100 μ M) induces marked oscillations in membrane potential that persist up to 30 min in the continuous presence of the ligand. These observations are in line with the finding that several hormones, including histamine, can induce Ca²⁺ oscillations in other cell types [39–43]. Each hyperpolarization is followed by a substantial depolarization and increased membrane resistance, suggesting either that [Ca²⁺]_i reaches a level below normal steady state or that an as yet unidentified ion channel is closed by mechanisms independent of [Ca²⁺]_i. As with the formation of inositol phosphates, the oscillations persist in the continued presence of histamine and cease immediately when the antagonist pyrrolamine is added.

A single injection of GTP[S] mimics the histamine-induced oscillations in membrane potential in both frequency and amplitude. These oscillations can be due

either to periodic changes in $\text{Ins}(1,4,5)\text{P}_3$ liberated [40] or to opening and closing of intracellular Ca^{2+} channels at a constant level of $\text{Ins}(1,4,5)\text{P}_3$ [44]. Since $\text{Ins}(1,4,5)\text{P}_3$ levels are only transiently elevated in histamine-stimulated HeLa cells, whereas membrane-potential oscillations continue for a much longer period, our data support a model in which a series of transient $\text{Ins}(1,4,5)\text{P}_3$ accumulations is generated as a result of feedback-inhibition by diacylglycerol-activated protein kinase C [40]. This notion is further supported by the observation that microinjections of $\text{Ins}(1,4,5)\text{P}_3$ are ineffective in generating oscillations.

Because each cell responds with different kinetics, oscillations in $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ are difficult to detect when populations of cells are studied. However, since $\text{Ins}(1,4,5)\text{P}_3$ accumulation reaches peaks at two different time points (4–7 and 30 s respectively; Fig. 5b), it is tempting to speculate that this phenomenon reflects the first two InsP_3 oscillations. Similar biphasic $\text{Ins}(1,4,5)\text{P}_3$ responses were recently reported for bradykinin-stimulated NG-115 cells [14].

In conclusion, we have shown that histamine, a growth factor and a chemoattractant for HeLa cells, triggers a prolonged breakdown of inositol lipids concomitant with sustained oscillations in membrane potential. Since these oscillations are most likely due to periodic changes in $[\text{Ca}^{2+}]_i$, our observations imply a prolonged activation of Ca^{2+} -dependent processes and stress the importance of Ca^{2+} in long-term events such as growth stimulation.

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REFERENCES

- Douglas, W. W. (1979) in *The Pharmacological Basis of Therapeutics* (Goodman, L. S. & Gilman, A., eds.), pp. 589–629, Macmillan, New York.
- Pounder, R. E. (1984) *Pharmacol. Ther.* **26**, 221–234.
- Bartholeyns, J. & Fozard, J. R. (1985) *Trends Biochem. Sci.* **10**, 123–125.
- Black, J. W., Duncan, W. A. H., Durant, C. J., Ganelli, C. R. & Parsons, M. E. (1972) *Nature (London)* **236**, 385–390.
- Arrang, J. M., Garbarg, M. & Schwartz, J. C. (1983) *Nature (London)* **302**, 832–837.
- Arrang, J. M., Garbarg, M., Lancelot, J. C., Lecomte, J. M., Pollard, H., Robba, M., Schunack, W. & Schwartz, J. C. (1987) *Nature (London)* **327**, 117–123.
- Hill, S. J. (1987) *Nature (London)* **327**, 104–105.
- Ishikawa, S. & Sperelakis, N. (1987) *Nature (London)* **327**, 158–160.
- Daum, P. R., Downes, C. P. & Young, J. M. (1984) *J. Neurochem.* **43**, 25–32.
- Claro, E., Garcia, A. & Picatoste, F. (1987) *Mol. Pharmacol.* **32**, 384–390.
- Nakahata, N. & Harden, T. K. (1987) *Biochem. J.* **241**, 337–344.
- Hegstrand, L. R., Kanof, P. D. & Greengard, P. (1976) *Nature (London)* **260**, 163–165.
- Tilly, B. C., Tertoolen, L. G. J., Remorie, R., Ladoux, A., Verlaan, I., de Laat, S. W. & Moolenaar, W. H. (1990) *J. Cell Biol.*, in the press.
- Tilly, B. C., van Paridon, P. A., Verlaan, I., Wirtz, K. W. A., de Laat, S. W. & Moolenaar, W. H. (1987) *Biochem. J.* **244**, 129–135.
- Jackson, T. R., Hallam, T. J., Downes, C. P. & Hanley, M. R. (1986) *EMBO J.* **6**, 49–54.
- Hansen, C. A., Mah, S. & Williamson, J. R. (1986) *J. Biol. Chem.* **261**, 8100–8103.
- Howell, T. W. & Gomperts, B. D. (1987) *Biochim. Biophys. Acta* **927**, 177–183.
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
- Moolenaar, W. H., Aerts, R. J., Tertoolen, L. G. J. & de Laat, S. W. (1986) *J. Biol. Chem.* **261**, 279–289.
- Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450.
- Yada, T. & Okada, Y. (1984) *J. Membr. Biol.* **77**, 33–44.
- Tertoolen, L. G. J., Tilly, B. C., Irvine, R. F. & Moolenaar, W. H. (1987) *FEBS Lett.* **214**, 365–369.
- Hepler, J., Nakahata, N., Lovenberg, T. W., DiGuseppi, J., Herman, B., Earp, H. S. & Harden, T. K. (1987) *J. Biol. Chem.* **262**, 2951–2956.
- Pike, L. J. & Eakes, A. T. (1987) *J. Biol. Chem.* **262**, 1644–1651.
- Tilly, B. C., van Paridon, P. A., Verlaan, I., de Laat, S. W. & Moolenaar, W. H. (1988) *Biochem. J.* **252**, 857–863.
- Sternweis, P. C. & Gillman, A. G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4888–4891.
- Northup, J. K., Smigel, M. D., Sternweis, P. C. & Gillman, A. G. (1983) *J. Biol. Chem.* **258**, 11369–11376.
- Katada, T., Bokoch, G. M., Smigel, M. P., Ui, M. & Gillman, A. G. (1984) *J. Biol. Chem.* **259**, 3586–3595.
- Bigay, J., Deterre, P., Pfister, C. & Chabre, M. (1985) *FEBS Lett.* **191**, 181–185.
- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215.
- Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Gimbrone, M. A. & Alexander, R. W. (1985) *J. Biol. Chem.* **260**, 14158–14162.
- Orellana, S. A., Solski, P. A. & Brown, J. M. (1985) *J. Biol. Chem.* **260**, 5236–5239.
- Leeb-Lundberg, L. M. F., Cotecchia, S., Lomasney, J. W., DeBernardis, J. F., Lefkowitz, R. J. & Caron, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5651–5655.
- Sauvé, R., Simoneau, C., Parent, L., Monette, R. & Roy, G. (1987) *J. Membr. Biol.* **96**, 199–208.
- Hazama, A., Yada, T. & Okada, Y. (1985) *Biochim. Biophys. Acta* **845**, 249–253.
- Tilly, B. C. & Moolenaar, W. H. (1988) in *Inositol Lipids in Cell Signalling* (Michell, R. H., Drummond, A. H. & Downes, C. P., eds.), pp. 485–499, Academic Press, London.
- Nakahata, N., Martin, M. W., Hughes, A. R., Hepler, J. R. & Harden, T. K. (1986) *Mol. Pharmacol.* **29**, 188–195.
- Irvine, R. F. & Moore, R. M. J. (1986) *Biochem. J.* **240**, 917–920.
- Morris, A. P., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. (1987) *Nature (London)* **330**, 653–655.
- Rapp, P. E. & Berridge, M. J. (1981) *J. Exp. Biol.* **93**, 119–132.
- Woods, N. M., Cuthbertson, K. S. R. & Cobbald, P. H. (1986) *Nature (London)* **319**, 600–603.
- Ambler, S. K., Poenie, M., Tsien, R. Y. & Taylor, P. (1988) *J. Biol. Chem.* **263**, 1952–1959.
- Libscombe, D., Madison, D. V., Poenie, M., Reuter, H., Tsien, R. Y. & Tsien, R. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2398–2402.
- Jacob, R., Merritt, J. E., Hallam, T. J. & Rink, T. J. (1988) *Nature (London)* **335**, 40–45.
- Wakui, M., Potter, B. V. L. & Peterson, O. H. (1989) *Nature (London)* **339**, 317–320.