Regulation of phosphoinositide hydrolysis induced by histamine and guanine nucleotides in human HeLa carcinoma cells

Calcium and pH dependence and inhibitory role of protein kinase C

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The regulation of phospholipase C has been investigated in both intact and streptolysin-O permeabilized human HeLa carcinoma cells. Stimulation of phospholipase C by histamine and guanosine-5'-O-thiotriphosphate (GTP[S]) requires the presence of at least 10 nM free Ca^{2+}, but is not significantly further increased by raising $[Ca^{2+}]_i$ to $> 10^{-6}$ M. The pH optimum of the inositol phosphate response is at pH 6.8, while small changes in intracellular pH, as occur during hormonal stimulation (0.2–0.4 unit) attenuate the histamine/GTP[S]-induced stimulation of phospholipase C. Increasing cellular cAMP levels, either through addition of cell permeable cAMP analogues to intact cells or by stimulation with isoproterenol, does not affect histamine responsiveness, arguing against cross-talk between both signalling pathways. In contrast, we found that the response to histamine and/or GTP[S] is largely inhibited after brief pretreatment of the cells with phorbol esters or synthetic diacylglycerol prior to permeabilization, suggesting that protein kinase C exerts feedback inhibition at the level of, or downstream from, the putative GTP-binding protein.

Histamine; Inositol lipid; Growth factor; Inositol phosphate; Phospholipase C

1. INTRODUCTION

Receptor-mediated hydrolysis of inositol phospholipids is among the earliest detectable cellular responses to such diverse stimuli as hormones, growth factors, neurotransmitters and light [1–3]. Activation of the receptor stimulates a phospholipase C, resulting in the cleavage of phosphoinositides with the formation of $Ins(1,4,5)P_3$, the second messengers involved in Ca^{2+}-signalling [4], and 1,2-diacylglycerol, the endogenous activator of protein kinase C [5]. It is now widely assumed that guanine-nucleotide binding regulatory proteins (G-proteins) are involved in the coupling between hormone receptor and phospholipase C, although their identity remains unknown [6–8].

We recently showed that histamine, a ubiquitously occurring local hormone involved in many important physiological responses, can function as a growth factor and chemo-attractant for several cell lines, including human HeLa carcinoma cells [9]. These responses are mediated through activation of H_1-type receptors, which couple to the inositol lipid signalling pathway. Indeed, inositol phosphate formation and subsequent changes in $[Ca^{2+}]_i$ and pH_i are among the first detectable responses in histamine-stimulated HeLa cells [9,10]. In this report, we investigate the effects of physiological changes in $[Ca^{2+}]_i$, pH_i, cAMP and activated protein kinase C on histamine-induced phosphoinositide hydrolysis. Our data indicate that, while Ca^{2+} and cAMP have no major effects, pH_i and protein kinase C are important parameters in regulating receptor-mediated activation of phospholipase C.

2. MATERIALS AND METHODS

2.1. *Materials*

1,2-Dioctanoyl-rac-glycerol (DiC8), histamine and O-tetradecanoyl phorbol 13-acetate (TPA) were obtained from Sigma Chem. Corp. Other agents were from the following sources: fetal calf serum from Hyclone, GTP[S] from Boehringer Mannheim and streptolysin-O from Wellcome Nederland BV. *myo*-[2-^{3}H]Inositol (12.3 Ci/mmol) was purchased from Amersham International.

2.2. *Cell culture*

Human HeLa carcinoma cells were routinely grown in Dulbecco's Minimal Essential Medium (DMEM) containing 7.5% (v/v) fetal calf serum. Nearly confluent cultures were labelled to near-isotopic equilibrium with [^{3}H]inositol in DMEM/Ham's F-12 medium (1:1, v/v) containing 10 μg/ml transferrin for 24 h.

2.3. *Stimulation of permeabilized HeLa cells*

Nearly confluent monolayers were permeabilized with streptolysin-

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O, essentially as described by Howell and Gomperts [11]. Briefly, cultures were incubated for 5 min in a buffer (137 mM NaCl, 2.7 mM KCl, 2 mM EGTA, 1 mM $CaCl_2$, 10 mM LiCl, 20 mM Pipes, 1 mg/ml bovine serum albumin, 5.6 mM glucose, 1 mM ATP; pH 6.8) containing 0.4 IU of streptolysin-O. After washing the cells twice with buffer (1 ml), hormone and GTP[S] were added. The incubations were terminated by adding trichloroacetic acid (1 ml, 10%), and an inositol phosphate containing fraction was prepared and analyzed on AG 1 × 8 (Biorad) columns as described previously [10].

2.4. *Separation of inositol phosphates by HPLC*

A fraction containing inositol phosphates was prepared as described above and separated on a Partisil Sax column (250 × 4.6 mm; Whatman) at a flow rate of 1.25 ml/min, as described previously. For quantification of the various inositol phosphates, 0.5 min (625 μl) fractions were collected and ^3H radioactivity was determined [10].

2.5. *Preparation of Ca^{2+}-buffers*

Calcium buffers were calculated and prepared as described by Howell and Gomperts [11] using a computer program based on the algorithm of Perrin and Sayce [12].

3. RESULTS

3.1. *Streptolysin-O as a permeabilizing agent*

Permeabilized cell preparations allow experimental control of intracellular compartments and, thereby, facilitate studying receptor-mediated phospholipase C activation. Human HeLa carcinoma cells were treated with streptolysin-O, a bacterial toxin known to permeabilize monolayers of cells [11]. Streptolysin-O treatment renders the cells leaky within a few minutes, as shown by the uptake of the membrane impermeable

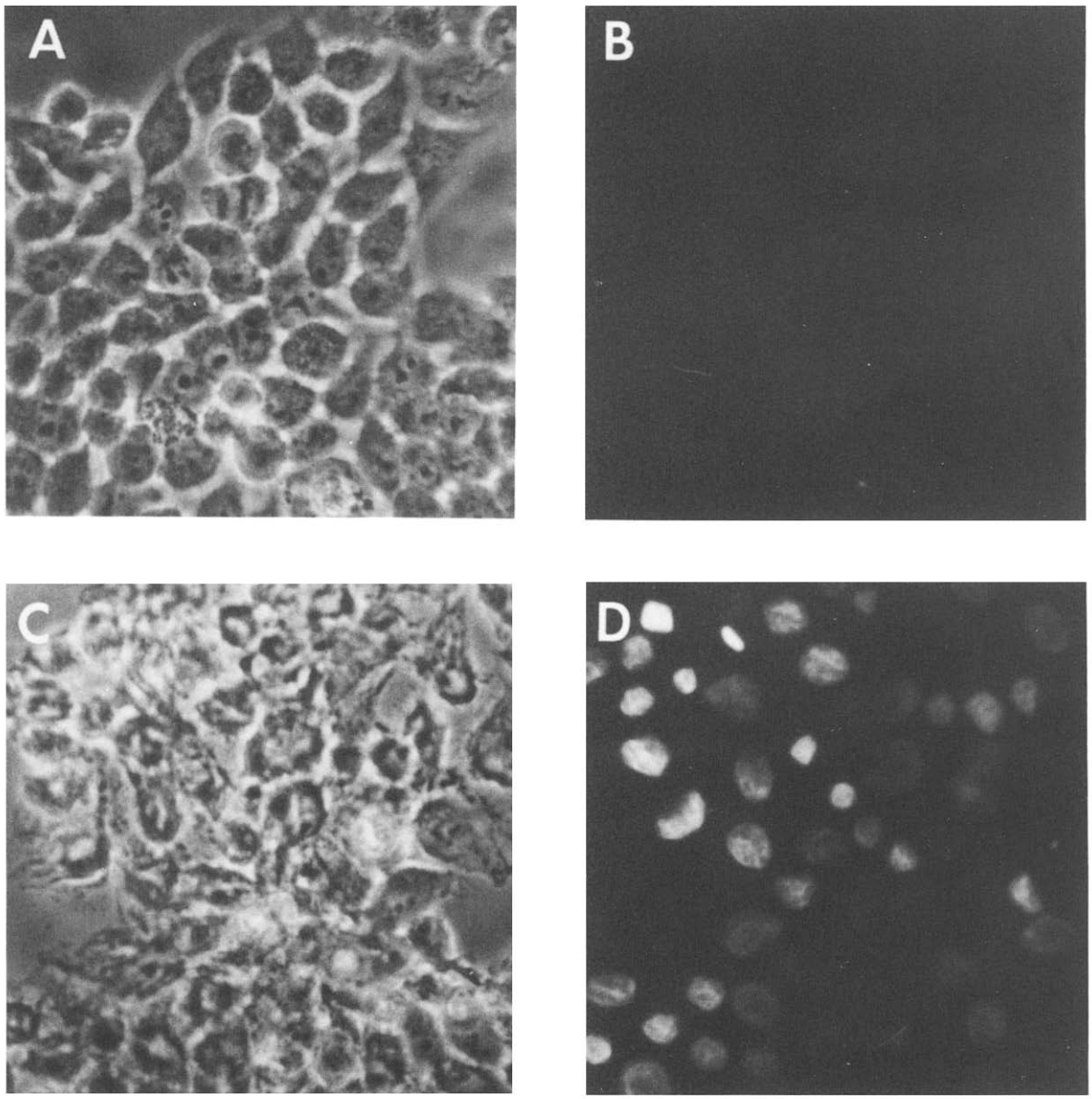

Fig. 1. Uptake of Hoechst 33258 into streptolysin-O permeabilized cells. Nearly confluent cultures of HeLa cells were treated with (C–D) or without (A–B) streptolysin-O (0.4 IU) for 5 min. Thereafter, the cultures were washed and Hoechst 33258 nuclear dye (0.5 μg/ml) was added. (A, C) Phase contrast image. (B, D) Fluorescence image (excitation wavelength: 370 nm; emission wavelength, 430 nm).

nuclear dye Hoechst 33258 (Fig. 1). Importantly, the cells maintain permeability after removal of the toxin for at least 30 min, while receptor-mediated inositol lipid hydrolysis remains intact. In contrast, when HeLa cells were permeabilized with saponin (0.005%), receptor-mediated phospholipase C activation was no longer detectable (results not shown).

3.2. *Calcium dependence of the histamine/GTP[S] response*

As reported previously, histamine and GTP[S] potently activate hydrolysis of inositol phospholipids in human HeLa carcinoma cells, concomitant with a transient biphasic rise in $[Ca^{2+}]_i$ and a steady increase in cytoplasmic pH_i [9,10]. To determine how intracellular Ca^{2+} modulates phospholipase C activity, streptolysin-O permeabilized cells were incubated in buffers of different *p*Ca and histamine-responsiveness was tested.

Phospholipase C activation by histamine and GTP[S] is strongly suppressed in nominally Ca^{2+}-free media containing 3 mM EGTA (Fig. 2). Raising the free concentration of Ca^{2+} (10 nM) restores the histamine/GTP[S]-induced formation of inositol phosphates completely, and no significant increase in phospholipase C activation was observed when $[Ca^{2+}]$ was progressively increased between 100 nM–1 μM, i.e., the physiological range. Even at a 10 μM free Ca^{2+}, far more than achieved through histamine stimulation in vivo, inositol phosphate levels were not significantly increased. These results indicate that although Ca^{2+} is essential for phospholipase C activation, physiological changes in $[Ca^{2+}]_i$ do not affect the magnitude of the PtdIns response. Comparison of the individual inositol phosphates formed at 100 nM and 10 μM Ca^{2+}, respectively, does not show a significant change in the levels of both $InsP_3$ isomers and $InsP_4$ (a small Ca^{2+}-dependent increase in InsP of approx. 30% was observed (results not shown)).

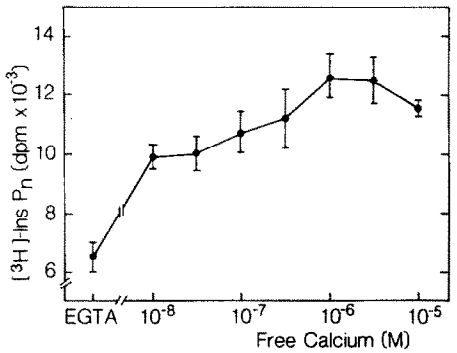

Fig. 2. Calcium-dependence of the histamine- and GTP[S]-induced inositol phosphate formation. Permeabilized HeLa cells were treated with histamine (100 μM) and GTP[S] (10 μM) for 10 min at various $[Ca^{2+}]$. Calcium buffers were prepared as described in section 2. A 'Ca^{2+}-free' buffer was made by replacing calcium for 3 mM EGTA. Data are expressed as means ± SE for 3 incubations. Asterisks indicate a significant difference ($P < 0.05$) relative to control cultures ($[Ca^{2+}]$ = 200 nM).

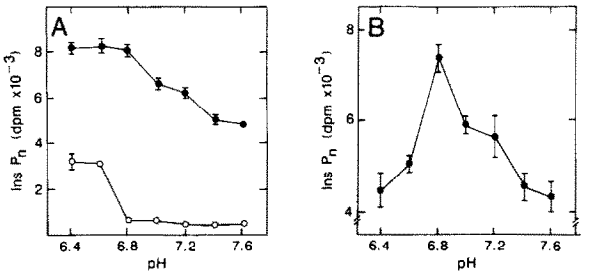

Fig. 3. Effects of pH_i on the histamine- and GTP[S]-induced accumulation of inositol phosphates. (A) [^{3}H]Inositol labelled cells were permeabilized and stimulated with histamine and GTP[S] (10 μM and 100 μM, respectively) at various pH_i. $[Ca^{2+}]_i$ was clamped at 200 nm. Open symbols represent control, closed symbols histamine + GTP[S]-stimulated cultures. (B) Calculated difference between histamine + GTP[S]-stimulated and control cultures at the indicated pH. Data are expressed as means ± SE for n = 3.

3.3. *Dependence on intracellular pH*

In addition to a transient rise in $[Ca^{2+}]_i$, histamine also induces a steady increase in cytoplasmic pH (pH_i) as a result of activation of the Na^+/H^+-exchanger [9]. The pH-dependence of the histamine/GTP[S]-induced activation of phospholipase C is shown in Fig. 3. When intracellular calcium is kept constant at 200 nM, the turn-over of inositol lipids in non-stimulated cells increases significantly as pH drops below 6.8 (Fig. 3A). Above pH 6.8 the basal rate of inositol phosphate formation remains constant, but the hormone/GTP[S]-induced activation of phospholipase C decreases progressively between pH 6.8 and 7.6 (Fig. 3A). From these data we then calculated the pH-dependence of $InsP_n$ accumulation in histamine/GTP[S]-stimulated cells (Fig. 3B). The pH optimum of the $InsP_n$ response is at 6.8, which is close to the steady state pH_i of intact HeLa cells [9], while the response is significantly reduced in both the lower and higher pH region.

3.4. *Lack of cross-talk with cAMP-generating pathway*

In some cell systems activators of adenyl cyclase and cAMP-analogues are potent inhibitors of inositol lipid breakdown [13–15], suggesting cross-talk between both signalling systems. We have tested both the effects of permeable cAMP analogues (db-cAMP, 1 mM; 8-bromo-cAMP, 0.5 mM) and isoproterenol (100 μM), a β-adrenergic agonist known to increase cAMP levels in HeLa cells [16,17, by pretreating intact cells for 2 h (cAMP-analogues) or 10 min (isoproterenol) prior to histamine stimulation. No effects were found on histamine-induced inositol phosphate formation (not shown), indicating absence of cross-talk. This notion is supported by our observation that cAMP, in the presence of ATP, does not affect the histamine/GTP[S] responsiveness in permeabilized HeLa cells.

3.5. *Effects of TPA on streptolysin-O-treated cells*

Treatment of intact HeLa cells with phorbol esters or synthetic diacylglycerols, completely blocks histamine-induced phospholipase C activity [10]. Also in permeabilized cells, the histamine-induced formation of inositol phosphates is inhibited by activators of protein kinase C. Fig. 4 shows that treating cells with TPA (100 ng/ml) or diC8 (50 μg/ml; not shown) during 15 min prior to permeabilization causes a decrease in inositol phopshate accumulation induced by histamine/GTP[S]. In contrast, when protein kinase C activity is down-regulated, by treating the cells with 100 ng/ml TPA for 18 h, the $InsP_n$ histamine/GTP[S] response is even somewhat enhanced. Under these conditions, cellular protein kinase C activity is reduced to about 15% of that in non-treated cells (G.T. Snoek, personal communication). Inositol phosphate formation by GTP[S] alone is also completely blocked after pretreatment with TPA (Fig. 4), suggesting that protein kinase C exerts a regulatory role at, or down-stream from, the putative G-protein.

4. DISCUSSION

Activation of the histamine H_1 receptor is known to result in the hydrolysis of polyphosphoinositides by phospholipase C, with concomitant production of several inositol phosphates and subsequent elevation of cytoplasmic free Ca^{2+}, as has been reported for various cell types including endothelial, smooth muscle and nervous tissue-derived cells [18–23]. Human HeLa cells provide a convenient system to explore H_1 receptor-mediated signal transduction in further detail. Previously, we have analyzed histamine-induced inositol phosphate generation, Ca^{2+}-signalling and membrane potential oscillations in HeLa cells [10].

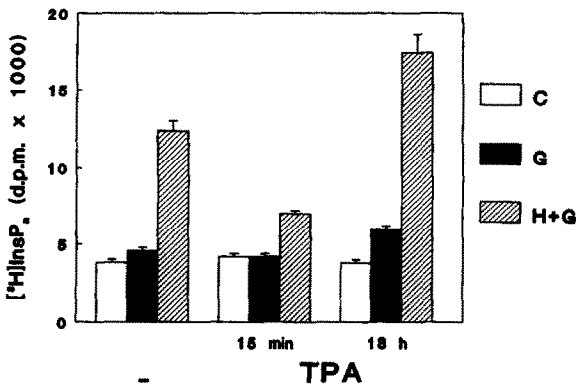

Fig. 4. Inhibition of inositol phosphate formation by TPA in permeabilized cells. [^{3}H]Inositol prelabelled cultures were treated with TPA (100 ng/ml; 15 min or 18 h) prior to permeabilization. Thereafter, inositol phosphate formation by histamine (H; 100 μM), GTP[S] (G; 10 μM) and histamine plus GTP[S] (H + G) was determined. Control incubations (C) are indicated. Data are expressed as means ± SE for triplicate cultures.

To study G-protein mediated phospholipase C activation in more detail, we have used the bacterial toxin streptolysin-O to permeabilize cells in monolayer. Using this method, direct access to the cytoplasmic compartments is achieved, while the H_1-receptor-linked phospholipase C signalling function appears to remain intact. It has been shown that the plasma membrane lesions generated by streptolysin-O are rather large (average diameter approx. 12 nm [11]), allowing rapid equilibration between extra- and intracellular compartments. Detergent-like agents such as saponin, although quite effective in permeabilizing HeLa cells, were found to severely impair receptor-linked inositol lipid hydrolysis. It should be noted that differences between intact and permeabilized HeLa cells do occur, especially with respect to kinetics and levels of inositol phosphates formed during histamine stimulation [10]. Nevertheless, the permeabilized cell system allows to gain further insight into the various parameters that determine phospholipase C activation.

Our results indicate that the histamine-induced inositol phosphate formation is insensitive to free $[Ca^{2+}]_i$ over the physiological range (100 nM–1 μM). However, a minimum of 10 nM $[Ca^{2+}]$ is required for normal responsiveness. These results are in line with the observations of Lew et al. [24] in HL-60 cells, but in contrast to those of others [25–27], who reported phospholipase C activity to be proportional to the free Ca^{2+} concentrations between 50–500 nM.

Interestingly, histamine-induced inositol phosphate formation is sensitive to small changes in cytoplasmic pH. The pH optimum of phospholipase C activity is near 6.8, which is close to the steady-state pH_i of intact HeLa cells [10], while changes in pH_i as small as 0.2–0.4 unit decrease the magnitude of the response by approx. 25%. This phenomenon may be of biological significance, since receptors that act through the phospholipase C/protein kinase C pathway mediate activation of the Na^+/H^+-exchanger [28], resulting in cytoplasmic alkalinization. Although the observed pH effects are rather small, the results from these in vitro experiments suggest a novel role for hormone-induced cytoplasmic alkalinization, namely attenuation of phospholipase C activity. Further experiments are required to further substantiate this hypothesis.

Activation of protein kinase C, by phorbol esters or synthetic diacylglycerol, strongly inhibit inositol phosphate formation in both intact and permeabilized HeLa cells. This result is in agreement with the rather universal negative feedback function of protein kinase C in uncoupling receptors from second messenger generating systems [29–31]. Interestingly, the GTP[S]-induced inositol phosphate formation is also blocked after pretreatment with TPA. These results suggest that protein kinase C mediated feedback inhibition occurs at the level of the putative G-protein, or, more downstream, at phospholipase C itself.

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