Regulation of histamine- and UTP-induced increases in Ins(1,4,5)P₃, Ins(1,3,4,5)P₄ and Ca²⁺ by cyclic AMP in DDT₁ MF-2 cells

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1 Stimulation of P_{2U}-purinoceptors with UTP or histamine H₁-receptors with histamine gave rise to the formation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) in DDT₁ MF-2 smooth muscle cells.

2 Stimulation of P_{2U}-purinoceptors or histamine H₁-receptors caused an increase in cytoplasmic Ca²⁺, consisting of an initial peak, representing the release of Ca²⁺ from internal stores and a sustained phase representing Ca²⁺ influx.

3 The P_{2U}-purinoceptor-mediated Ca²⁺-entry mechanism was more sensitive to UTP than Ca²⁺-mobilization (EC₅₀: 3.3 µM ± 0.4 µM vs 55.1 µM ± 9.2 µM), in contrast to these processes activated by histamine H₁-receptors (EC₅₀: 5.8 µM ± 0.6 µM vs 3.1 µM ± 0.5 µM).

4 Pre-stimulation of cells with several adenosine 3':5'-cyclic monophosphate (cyclic AMP) elevating agents, reduced the histamine H₁-receptor-mediated formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. Forskolin completely inhibited Ins(1,4,5)P₃ formation (IC₅₀: 158 ± 24 nM) whereas Ins(1,3,4,5)P₄ formation was inhibited by only 45% (IC₅₀: 173 ± 16 nM). The P_{2U}-purinoceptor-mediated production of these inositol phosphates was not affected by cyclic AMP.

5 Forskolin and isoprenaline reduced the histamine-induced increase in cytoplasmic Ca²⁺, as measured in Ca²⁺ containing medium and in nominally Ca²⁺-free medium but did not change the UTP-induced increase in cytoplasmic Ca²⁺.

6 These results clearly demonstrate that cyclic AMP differentially regulates components of the histamine induced phospholipase C signal transduction pathway. Furthermore, cyclic AMP does not affect the phospholipase C pathway activated by stimulation of P_{2U}-purinoceptors in DDT₁ MF-2 cells.

Keywords: Ins(1,4,5)P₃; Ins(1,3,4,5)P₄; Ca²⁺; cyclic AMP; UTP; histamine; DDT₁ MF-2 cell

Introduction

Stimulation of mammalian cells with a wide variety of agonists, acting on cell surface receptors leads, via heterotrimeric regulatory G-proteins, to activation of phospholipase C resulting in the formation of the second messengers diacylglycerol, inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄). The role of Ins(1,4,5)P₃ in the release of Ca²⁺ from intracellular structures by binding to specific receptors is well established (Berridge & Irvine, 1989), whereas a role for Ins(1,3,4,5)P₄ in this process was proposed more recently (Elly *et al.*, 1990; Hoiting *et al.*, 1990; Gawler *et al.*, 1991; Molleman *et al.*, 1991).

In smooth muscle cells the receptor stimulated increase in the cytoplasmic Ca²⁺ concentration leads to an initial phase of contraction of the intact tissue mediated by Ca²⁺/calmodulin-dependent myosin light chain kinases (Kamm & Stull, 1985). Stimulation of receptors activating adenylyl cyclase or direct activation of adenylyl cyclase by forskolin (Seamon & Daly, 1986) gives rise to the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP), a concomitant activation of protein kinase A and relaxation of the smooth muscle cells (Abdel-Latif, 1991). Several pathways are suggested for protein kinase A to inhibit contraction of smooth muscle, including effects on Ca²⁺ metabolism: (1) inhibition of phosphoinositide hydrolysis and Ins(1,4,5)P₃ production (Hall *et al.*, 1989; Manolopoulos *et al.*, 1991); (2) phosphorylation of the Ins(1,4,5)P₃ receptors resulting in

diminished Ca²⁺ release from intracellular stores (Xuan *et al.*, 1991; Tang *et al.*, 1992); (3) enhanced Ca²⁺ uptake into intracellular stores (Mueller & van Breemen, 1979); (4) stimulation of Ca²⁺ extrusion (Bülbring & den Hertog, 1980); (5) inhibition of Ca²⁺ influx into cells (Bülbring & Tomita, 1987; Chen & Rembold, 1992). Also, phosphorylation of myosin light chain kinase by protein kinase A reduces its affinity for the Ca²⁺/calmodulin complex, thereby inhibiting myosin light chain phosphorylation and contraction (Adelstein *et al.*, 1978).

We have shown previously that stimulation of histamine H₁-receptors or P₂-purinoceptors on DDT₁ MF-2 smooth muscle cells, derived from a Syrian hamster vas deferens, leads to the formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, release of Ca²⁺ from intracellular stores and influx of Ca²⁺ across the plasma membrane (Hoiting *et al.*, 1990; Molleman *et al.*, 1989; 1991). The P₂-purinoceptors were recently identified as P_{2U}-purinoceptors (Van der Zee *et al.*, 1992). Remarkably, stimulation of DDT₁ MF-2 cells with histamine or nucleotides results in Ca²⁺ release from partly different stores (Den Hertog *et al.*, 1992). Others observed a diminished accumulation of cytoplasmic Ca²⁺ after stimulation of histamine H₁-receptors (Dickenson *et al.*, 1993) or α₁-adrenoceptors in the presence of cyclic AMP (Schachter *et al.*, 1992) and a cyclic AMP-mediated reduced formation of inositol phosphates (Gerwins *et al.*, 1992; Dickenson *et al.*, 1993).

We show here that cyclic AMP differentially affects the histamine-mediated formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ and that cyclic AMP regulates the activation of the phospholipase C pathway mediated by histamine H₁-receptors but not by P_{2U}-purinoceptors in DDT₁ MF-2 cells.

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Methods

Cell culture

DDT₁ MF-2 cells, derived from a Syrian hamster vas deferens (Norris *et al.*, 1974) were cultured in Dulbecco's modified essential medium supplemented with 7 mM NaHCO₃, 10 mM HEPES at pH 7.2 (DMEM) and 10% foetal calf serum at 37°C in 5% CO₂ (Molleman *et al.*, 1989).

Preparation of samples for cyclic AMP, *Ins*(1,4,5)*P*₃, and *Ins*(1,3,4,5)*P*₄ measurements

DDT₁ MF-2 cells were grown in monolayers in 9.6 cm² plastic wells as described earlier (Hoiting *et al.*, 1990). The medium was replaced by 2 ml DMEM at 20°C 30 min before starting the experiment by adding agonists. Before adding P_{2U}-purinoceptor- or histamine H₁-receptor agonists, some cell preparations were pre-exposed to forskolin (1 µM) for 10 min or to isoprenaline (10 µM, 10 min) and phentolamine (2 µM, 10 min) to avoid α-adrenoceptor specific events. Cimetidine (10 µM, 5 min in advance) was added to avoid histamine H₂-receptor-mediated events. After removing the medium, reactions were stopped with 400 µM 5% trichloroacetic acid (TCA) and placed on ice for at least 45 min.

Samples were washed 3 times with 800 µl water saturated diethylether and neutralised with KOH (25 µl, 0.2 M).

Measurements of cyclic AMP

A radioligand binding assay was used to determine cyclic AMP concentrations, using a standard curve of cyclic AMP

in ether extracted TCA solution (Brown *et al.*, 1971). To reach equilibrium, 50 µl sample was incubated with a buffer composed of 50 mM Tris-HCl (pH 7.4), 4 mM EDTA, 20 µg cyclic AMP binding protein, 200 µg bovine serum albumin and 10 µl [³H]-cyclic AMP (190 nM, 40 Ci mmol⁻¹) to a total volume of 160 µl at 4°C for at least 2 h. The reaction was terminated by adding 500 µl of a charcoal suspension (Norit A special, 3.5 g l⁻¹) followed by centrifugation to remove the excess [³H]-cyclic AMP. Radioactivity in the supernatant was measured by scintillation counting.

Measurements of *Ins*(1,4,5)*P*₃

Mass measurements of *Ins*(1,4,5)*P*₃ were performed as described earlier, using a standard curve of *Ins*(1,4,5)*P*₃ in ether-extracted TCA solution (Molleman *et al.*, 1991). In brief, samples were assayed in 25 mM Tris/HCl (pH = 9.0), 1 mM EDTA, 1 mg bovine serum albumin, [³H]-*Ins*(1,4,5)*P*₃ (3.3 Ci mmol⁻¹, 2000 c.p.m./assay) and about 1 mg binding protein for 15 min. The binding protein was isolated from fresh beef liver (Chilvers *et al.*, 1989). Bound and free radioactivity were separated by centrifugation. The radioactivity in the pellet was determined by scintillation counting.

Measurements of *Ins*(1,3,4,5)*P*₄

Binding protein for *Ins*(1,3,4,5)*P*₄ was prepared from pig cerebellum as described earlier by Donié & Reiser (1989). A standard curve of *Ins*(1,3,4,5)*P*₄ in ether-extracted TCA solution was used to determine *Ins*(1,3,4,5)*P*₄ contents. Samples were assayed for 20 min at 0°C in a mixture (total volume 80 µl) containing 1 mM EDTA, 25 mM sodium acetate

Figure 1 Forskolin and isoprenaline induced formation of cyclic AMP in DDT₁ MF-2 cells. The time-dependent increase in the intracellular cyclic AMP level elicited by (a) forskolin (1 µM) or (b) isoprenaline (10 µM). The concentration-dependent formation of cyclic AMP evoked by exposure of cells to (c) forskolin or (d) isoprenaline for 10 min. Each point represents the mean ± s.e. mean of 4 experiments.

(pH 5.0), 25 mM potassium phosphate, bovine serum albumin (2.5 mg ml⁻¹), Ins(1,3,4,5)P₄ binding protein (150 µg) and [3-³²P]-Ins(1,3,4,5)P₄ (5000 c.p.m./assay). Membrane bound ligand was separated from free-ligand by centrifugation (10,000 g, 2 min). Radioactivity in the pellet was determined by scintillation counting. [3-³²P]-Ins(1,3,4,5)P₄ was obtained after phosphorylation of Ins(1,4,5)P₃ with γ³²P-ATP of high specific activity (3000 Ci mmol⁻¹), using a recombinant rat brain Ins(1,4,5)P₃-kinase expressed in *Escherichia coli* as described previously (Takazawa *et al.*, 1990).

Measurements of intracellular calcium

Intracellular concentrations were measured as described earlier (Hoiting *et al.*, 1990). Cells (10⁶ cells ml⁻¹) suspended in DMEM containing 10% foetal calf serum were loaded with Indo-1 AM (1.5 µM) for 45 min at 37°C. The cells were collected by centrifugation (5 min, 1000 g) and washed two times before the fluorescence measurement with a buffer solution containing (mM): NaCl 145, KCl 5, MgSO₄ 0.5, CaCl₂ 1, glucose 10, HEPES 10 (pH 7.4) (Hesketh *et al.*, 1983). Calcium-free solution contained Mg²⁺ (6.2 mM) to prevent membrane leakage and EGTA (0.4 mM) to remove extracellular Ca²⁺ (Den Hertog, 1981). Indo-1 fluorescence of the cells (excitation: 325 nm; emission 400 nm and 480 nm) was measured at 22°C. Forskolin or isoprenaline and phenolamine were added 10 min before stimulation of histamine H₁-receptors or nucleotide receptors of cells, mounted in the fluorescence spectrophotometer. The cell suspension was continuously magnetically stirred. The internal calcium concentration was calculated (Hesketh *et al.*, 1983) using 0.015% of Triton X-100 as permeabilizing agent.

Statistics

Data are represented as mean ± s.e.mean. Student's unpaired *t* test was used to determine whether data were significantly different from control values. A sigma plot logistic curve fit programme (Jandel Scientific, U.S.A.) was used to determine EC₅₀ values and to analyse binding parameters obtained from the respective radioligand binding assays.

Chemicals

Adenosine 3':5'-cyclic monophosphate (cyclic AMP), adenosine 5'-O-(3-thiotrisphosphate) (ATPγS), inositol 1,4,5-trisphosphate sodium salt, inositol 1,3,4,5-tetrakisphosphate sodium salt, 8'-bromo-cyclic AMP and hexokinase were obtained from Boehringer (Germany). Adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) were obtained from Serva (Germany). Pertussis toxin (PTX), cimetidine, isoprenaline, forskolin and 3-isobutyl-1-methyl-xanthine were purchased from Sigma (U.S.A.). Histamine dihydrochloride was obtained from Fluka (Switzerland). Indo-1 AM was from Molecular probes (U.S.A.) and [2,8-³H]-adenosine 3':5'-cyclic monophosphate, D-[2-³H]-inositol 1,4,5-trisphosphate from Du Pont-New England Nuclear (U.S.A.).

Results

Activation of adenylyl cyclase

Stimulation of DDT₁ MF-2 cells with forskolin (1 µM) leads to a fourfold increase in the basal cellular cyclic AMP level

Figure 2 The effect of cyclic AMP on the histamine-induced formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ in DDT₁ MF-2 cells. The time-dependent formation of (a) Ins(1,4,5)P₃ and (b) Ins(1,3,4,5)P₄ evoked on stimulation with histamine (100 µM) of non-pretreated cells (○) and forskolin (1 µM, 10 min) pretreated cells (●). The formation of (c) Ins(1,4,5)P₃ and (d) Ins(1,3,4,5)P₄ evoked by stimulation of non-pretreated cells (○) and forskolin (1 µM, 10 min) pretreated cells (●) with different concentrations of histamine for 1 min. Data are expressed as the means ± s.e.mean of 5 or more experiments. Pretreated significantly different from non-pretreated; **P* < 0.05; ***P* < 0.01.

within 10 min (Figure 1a). Exposure of cells to isoprenaline (10 μ M, 10 min) results in a much more pronounced increase in the cyclic AMP level (Figure 1b). Cellular cyclic AMP showed a constant rise at forskolin concentrations in the range of 0.1 μ M–10 μ M (Figure 1c). A sharp rise in cyclic AMP was observed at higher cyclic AMP concentrations, possibly caused by occupation of a low affinity site for forskolin (Cussac *et al.*, 1993). Intracellular cyclic AMP was maximally enhanced by isoprenaline at a concentration of 1 μ M (EC_{50} : 34.6 nM $\pm$ 0.6; Figure 1d).

Cyclic AMP affects the histamine-induced phospholipase C pathway

The effect of pretreatment of DDT₁ MF-2 cells with forskolin (1 μ M, 10 min) or isoprenaline (10 μ M, 10 min) on the histamine H_1 -receptor-induced phospholipase C pathway was investigated. Cimetidine (10 μ M) was used to block histamine H_2 -receptors. Specific radioligand binding assays were used to quantify Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ accumulation. The increase in Ins(1,4,5)P₃ was totally inhibited in forskolin pre-stimulated cells (Figure 2a,c). Ins(1,3,4,5)P₄ formation was completely blocked during the first 30 s of histamine stimulation and was only partly blocked after 1 min in forskolin pre-exposed cells (Figure 2c,d). Pre-exposure of cells to isoprenaline (1 μ M, 10 min) or a phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (200 μ M, 10 min, 11.5 $\pm$ 1.9 pmol cyclic AMP per 10⁶ cells) or a cyclic AMP analogue, 8'-bromo-cyclic AMP (1 mM, 30 min) likewise caused inhibition of histamine-induced formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ (Table 1). The inhibitory effects of forskolin showed similar concentration-dependencies for Ins(1,4,5)P₃ (IC_{50} : 158 μ M $\pm$ 24 μ M) and Ins(1,3,4,5)P₄ (IC_{50} : 173 μ M $\pm$ 16 μ M) production (Figure 3). However, forskolin can totally abolish Ins(1,4,5)P₃ formation, while about 55% of histamine induced Ins(1,3,4,5)P₄ formation measured after 1 min is not sensitive to enhanced cyclic AMP.

It can be expected that the histamine H_1 -receptor-mediated increase in cytoplasmic Ca²⁺ ([Ca²⁺]_i) is also negatively regulated by intracellular cyclic AMP. Therefore, histamine-induced elevations of [Ca²⁺]_i were measured by Indo-1 fluorescence in Ca²⁺ containing medium and in nominally Ca²⁺-free medium. The effects of pretreatment of cells with forskolin or isoprenaline on basal [Ca²⁺]_i and on [Ca²⁺]_i achieved after agonist stimulation are shown as representative tracings in Figure 4 and summarized in Table 2. Not only the peak increase in [Ca²⁺]_i, but also the sustained increase measured 2 min after stimulation with histamine, caused exclusively by influx of Ca²⁺ from the extracellular environment (Molleman *et al.*, 1991), is reduced by pretreatment of cells with forskolin or isoprenaline (Figure 4, Table 2). In Ca²⁺-free medium forskolin and isoprenaline likewise attenuated the histamine-induced maximal accumulation of [Ca²⁺]_i (Figure 4, Table 2). The effects of enhanced cyclic AMP on inositol phosphates and [Ca²⁺]_i accumulation were all dependent on the histamine concentration as summarized in Table 3. Neither forskolin, isoprenaline, phentolamine (2 μ M), added to exclude α_1 -adrenoceptor activation by

Figure 3 Forskolin differentially inhibits histamine-induced Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ formation. Cells were pre-exposed to different concentrations of forskolin for 10 min before stimulation with histamine (100 μ M, 1 min). Data are represented as percentage of the histamine-induced stimulation in the absence of forskolin. Ins(1,4,5)P₃ (○), Ins(1,3,4,5)P₄ (●). Control basal and stimulated levels are presented in Table 1. Data are expressed as means $\pm$ s.e.mean of 6 experiments.

Figure 4 The effect of cyclic AMP on the histamine-induced increase in [Ca²⁺]_i in DDT₁ MF-2 cells. Before stimulation with histamine (100 μ M) cells were either not pretreated (a,d) or pre-exposed to forskolin (1 μ M, 10 min, b,e) or to isoprenaline (1 μ M, 10 min, c,f). Tracings are representative of at least 4 experiments performed in the presence (a,b,c) or absence (d,e,f) of extracellular Ca²⁺. Horizontal bars indicate the presence of histamine.

Table 1 The effect of several cyclic AMP elevating agents on basal and histamine-stimulated Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ in DDT₁ MF-2 cells

Treatment	Ins(1,4,5)P ₃ (pmol/10 ⁶ cells)		Ins(1,3,4,5)P ₄ (pmol/10 ⁶ cells)	
	Basal	Stimulated	Basal	Stimulated
Histamine	4.2 $\pm$ 0.3	6.3 $\pm$ 0.3	1.3 $\pm$ 0.1	4.2 $\pm$ 0.3
Histamine + isoprenaline	4.1 $\pm$ 0.4	4.5 $\pm$ 0.5*	1.4 $\pm$ 0.2	2.8 $\pm$ 0.3*
Histamine + IBMX	3.6 $\pm$ 0.3	4.5 $\pm$ 0.8*	1.3 $\pm$ 0.2	2.9 $\pm$ 0.1*
Histamine + 8'-bromo cyclic AMP	4.7 $\pm$ 0.4	4.7 $\pm$ 0.4**	1.1 $\pm$ 0.1	2.4 $\pm$ 0.2**

Cells were pretreated with isoprenaline (1 μ M, 10 min), 3-isobutyl-1-methyl-xanthine (200 μ M, 10 min) or 8'-bromo cyclic AMP (1 mM, 30 min) before stimulation with histamine (100 μ M, 1 min) and Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were measured. Data are expressed as means $\pm$ s.e.means of 6 experiments. Significantly different from control stimulation * P < 0.05; ** P < 0.01.

Table 2 The effect of adenylyl cyclase stimulation on histamine- and UTP-induced increases in $[Ca^{2+}]_i$ in DDT₁ MF-2 cells

Treatment	$[Ca^{2+}]_i$ (nM) extracellular Ca^{2+} present			$[Ca^{2+}]_i$ (nM) no extracellular Ca^{2+} present		
	Basal	Peak increase	2 min	Basal	Peak increase	
Histamine	163 ± 5	170 ± 17	23 ± 2	79 ± 4	104 ± 14	
Histamine + forskolin	160 ± 6	115 ± 20*	12 ± 2*	71 ± 3	46 ± 3*	
Histamine + isoprenaline	165 ± 4	111 ± 14*	12 ± 2*	69 ± 4	53 ± 4*	
UTP	169 ± 6	182 ± 11	63 ± 3	75 ± 3	115 ± 9	
UTP + forskolin	161 ± 5	204 ± 13	64 ± 4	71 ± 3	117 ± 6	
UTP + isoprenaline	173 ± 7	179 ± 7	64 ± 3	71 ± 3	110 ± 9	

Cells were pretreated with forskolin (1 μ M, 10 min) or isoprenaline (1 μ M, 10 min). Stimulated Ca^{2+} levels are given at their peak value and 2 min after stimulation with histamine (100 μ M) or UTP (1 mM). Data are expressed as means $\pm$ s.e.mean of 4 or more experiments. Significantly different from control stimulation. * P < 0.01.

Figure 5 The effect of cyclic AMP on the UTP-induced formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ in DDT₁ MF-2 cells. The time dependent formation of (a) Ins(1,4,5)P₃ and (b) Ins(1,3,4,5)P₄ evoked by stimulation with UTP (100 μ M) of non-pretreated cells (○) and forskolin (1 μ M, 10 min) pretreated cells (●). The formation of (c) Ins(1,4,5)P₃ and (d) Ins(1,3,4,5)P₄ evoked by stimulation of non-pretreated cells (○) and forskolin (1 μ M, 10 min) pretreated cells (●) with different concentrations of UTP for 1 min. Data are expressed as the mean $\pm$ s.e.mean of 5 or more experiments.

isoprenaline, nor cimetidine gave rise to accumulation of $[Ca^{2+}]_i$ themselves (not shown).

Cyclic AMP and the P_{2U} -purinoceptor activated phospholipase C pathway

In order to investigate the effect of an enhanced cellular cyclic AMP level on the P_{2U} -purinoceptor-induced phospholipase C pathway, DDT₁ MF-2 cells were either pretreated with forskolin (1 μ M, 10 min) or with isoprenaline (10 μ M, 10 min) before exposure to agonists of the P_{2U} -purinoceptor. In this study, UTP was used as P_{2U} -purinoceptor agonist in order to avoid undesirable influences of breakdown products of ATP, in particular adenosine. Adenosine has been shown to act synergistically on ATP-

induced phosphoinositide hydrolysis and enhancement of $[Ca^{2+}]_i$ in DDT₁ MF-2 cells (Gerwins *et al.*, 1992). Neither the time course nor the maximum value of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ production upon stimulation with UTP (100 μ M) were significantly altered by an enhanced cyclic AMP level (Figure 5a,b). Similar results were obtained when the non hydrolysable ligand, ATP γ S (100 μ M), was used as a P_{2U} -purinoceptor agonist (not shown). UTP-induced concentration-dependent accumulation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ was also not affected by pretreatment of cells with forskolin (Figure 5c,d, Table 3).

Similar negative results were obtained for the UTP-induced accumulation of $[Ca^{2+}]_i$. Both Ca^{2+} release and Ca^{2+} entry were unaffected by forskolin or isoprenaline pretreatment (Tables 2,3). Stimulation of DDT₁ MF-2 cells with ATP

Table 3 The effect of adenylyl cyclase stimulation on EC₅₀ values of histamine- and UTP-induced inositol phosphate formation and Ca²⁺ metabolism in DDT₁ MF-2 cells

	Histamine (EC ₅₀ μ M)			UTP (EC ₅₀ μ M)		
	Control	Forskolin	Isoprenaline	Control	Forskolin	Isoprenaline
Ins(1,4,5)P ₃	46.1 $\pm$ 0.9	>1000*	ND	74.3 $\pm$ 0.9	68.4 $\pm$ 3.0	ND
Ins(1,3,4,5)P ₄	26.3 $\pm$ 2.7	48.5 $\pm$ 4.2*	ND	138 $\pm$ 7	141 $\pm$ 7	ND
Ca ²⁺ -release	3.1 $\pm$ 0.5	6.2 $\pm$ 1.2*	ND	55.1 $\pm$ 9.2	92.7 $\pm$ 25.1	ND
Ca ²⁺ -entry	5.8 $\pm$ 0.6	11.7 $\pm$ 1.5*	13.4 $\pm$ 0.9*	3.3 $\pm$ 0.4	3.9 $\pm$ 0.6	3.1 $\pm$ 0.2
Maximal [Ca ²⁺] _i	5.0 $\pm$ 0.2	14.0 $\pm$ 0.8*	15.9 $\pm$ 1.5*	6.6 $\pm$ 0.3	6.9 $\pm$ 1.0	5.8 $\pm$ 0.7

EC₅₀ values are obtained after logistic curve fitting and presented as best fitted value $\pm$ s.e.mean. Cells were stimulated with histamine or UTP after pretreatment with forskolin (1 μ M, 10 min) or isoprenaline (10 μ M, 10 min). Ca²⁺-release was measured in the absence of extracellular Ca²⁺. Ca²⁺-entry represents the increase in [Ca²⁺]_i obtained after 2 min of agonist stimulation in Ca²⁺-containing medium. Maximal [Ca²⁺]_i represents the peak [Ca²⁺]_i obtained in the presence of extracellular Ca²⁺. ND: not determined. Significantly different from control: **P* < 0.01.

resulted in a similar concentration-dependent elevation of [Ca²⁺]_i as stimulation with UTP. Pre-exposure of the cells with forskolin did not affect the ATP induced increase in [Ca²⁺]_i (not shown).

Discussion

In this study the effect of cellular cyclic AMP on histamine H₁-receptor and P_{2U}-purinoceptor-mediated formation of inositol phosphates and increase in [Ca²⁺]_i was investigated in DDT₁ MF-2 smooth muscle cells. In order to enhance cellular cyclic AMP levels, two different methods were used. Forskolin, which acts directly on the catalytic subunit of adenylyl cyclase (Seamon & Daly, 1986) gave rise to a four-fold increase in the basal cyclic AMP level, whereas the β -adrenoceptor agonist, isoprenaline, enhanced the cyclic AMP level to a much larger extent. Apparently isoprenaline is more effective than forskolin at the concentrations used in activating the formation of cyclic AMP as measured by cyclic AMP mass in contrast to the equipotent action of these agents found by determination of the *de novo* synthesis of cyclic AMP in DDT₁ MF-2 cells (Dickenson *et al.*, 1993).

Exposure of DDT₁ MF-2 cells to histamine leads to the production of inositol phosphates, the release of Ca²⁺ from intracellular compartments and entry of Ca²⁺ from the external environment (Molleman *et al.*, 1991). These events are histamine H₁-receptor specific (Molleman *et al.*, 1991; White *et al.*, 1993). Enhanced cyclic AMP produced with forskolin or isoprenaline, as well as elevated cyclic AMP achieved by other means (by inhibiting cyclic AMP breakdown with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine and pre-exposure of cells to 8'-bromo cyclic AMP) was effective in blocking the histamine-induced production of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. The formation of Ins(1,4,5)P₃ and the formation of Ins(1,3,4,5)P₄ during the first 30 s of stimulation were completely abolished in forskolin pretreated cells. However, while Ins(1,4,5)P₃ formation is completely blocked for at least 5 min, Ins(1,3,4,5)P₄ formation was inhibited by only 45% after 1 min of histamine stimulation. The forskolin concentration-dependency for inhibition was similar for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ formation. These results suggest, that histamine induces cyclic AMP-sensitive formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ and that histamine stimulates a slowly evolving, cyclic AMP insensitive and Ins(1,4,5)P₃-independent pathway of Ins(1,3,4,5)P₄ production. It is not clear from where this Ins(1,3,4,5)P₄ originates. It has been shown previously in permeabilized cells or in purified enzyme systems, that the Ins(1,4,5)P₃ 3-kinase exhibits a very high affinity and low capacity for Ins(1,4,5)P₃ and is activated by increased [Ca²⁺]_i (Biden *et al.*, 1988; Morris *et al.*, 1988). Thus, another possible explanation of our results is that Ins(1,4,5)P₃ is phosphorylated immediately and at a high rate in DDT₁ MF-2 cells, especially under those conditions when production of Ins(1,4,5)P₃ is reduced.

A similar discrepancy between phorbol ester-induced inhibition of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ production was observed in adrenal chromaffin cells, though Ins(1,4,5)P₃ production was not completely blocked in this case (Boarder *et al.*, 1992). In some studies a stimulatory action of cyclic AMP on partly purified Ins(1,4,5)P₃ 3-kinase has been established (Biden *et al.*, 1988; Sim *et al.*, 1990). We also observed a minor activation of Ins(1,4,5)P₃ 3-kinase in forskolin pretreated cells as measured in permeabilized DDT₁ MF-2 cells (unpublished observation). However, activation of Ins(1,4,5)P₃ 3-kinase by cyclic AMP seems not to be prominent in intact cells, since the ratio Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ is not changed in forskolin pretreated cells after stimulation with UTP.

It should be noticed, that in pretreated cells, in parallel with Ins(1,3,4,5)P₄ formation, internal Ca²⁺ release and Ca²⁺-entry is observed under conditions where no Ins(1,4,5)P₃ is formed, suggesting that under these circumstances Ins(1,3,4,5)P₄ formation plays an important role in the enhancement of intracellular Ca²⁺ in DDT₁ MF-2 cells.

Forskolin and isoprenaline were equally effective in diminishing the histamine H₁-receptor-induced release of Ca²⁺ from intracellular stores and the entry of Ca²⁺, though isoprenaline increased the cellular cyclic AMP level to a much larger extent than forskolin. Apparently, a small increase in cellular cyclic AMP already elicits a full effect on the histamine-induced phospholipase C pathway. Similar results, describing the influence of cyclic AMP on total inositol phosphate formation and release of stored Ca²⁺ have been described (Dickenson *et al.*, 1993). An explanation for these results might be that cyclic AMP modulates the filling state of the histamine-sensitive Ca²⁺ store or the Ca²⁺-pump activity in the plasma membrane, thereby modulating phospholipase C activity. However, it has been found that the production of inositol phosphates is not dependent on Ca²⁺-release from internal stores (Den Hertog *et al.*, 1992) or on Ca²⁺-entry from the extracellular space (Schachter *et al.*, 1992) in DDT₁ MF-2 cells. This means that the diminished production of inositol phosphates cannot be explained by this model. A more reliable explanation is that cyclic AMP-activated protein kinase A exerts its effect on the histamine-induced activation of phospholipase C or prior to this point at the G-protein and/or receptor level.

The EC₅₀ values obtained for the histamine-induced formation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ are remarkably higher than those found for the histamine-evoked release of Ca²⁺ or Ca²⁺-entry, suggesting that a small and local production of inositol phosphates is sufficient to elicit the emptying of histamine sensitive Ca²⁺-pools.

Stimulation of DDT₁ MF-2 cells with ATP also gives rise to production of inositol phosphates and an increase in [Ca²⁺]_i (Molleman *et al.*, 1989; Hoiting *et al.*, 1990; Gerwins *et al.*, 1992). ATP and UTP share about the same potency for these P₂-purinoceptor-activated processes (Van der Zee *et al.*, 1992), thus classifying this receptor as a P_{2U}-purinoceptor

(Fredholm *et al.*, 1994). Forskolin did not affect the UTP or ATP γ S-induced generation of Ins(1,4,5)P $_3$ and Ins(1,3,4,5)P $_4$. In accordance with this, the related increase in [Ca $^{2+}$] $_i$, originating from release of stored Ca $^{2+}$ and from Ca $^{2+}$ -entry was not affected by cyclic AMP. These results show that cyclic AMP does not interfere with P $_{2U}$ -purinoceptor-mediated phospholipase C activation.

Surprisingly, it was found that the EC $_{50}$ values of the UTP-induced accumulation of Ins(1,4,5)P $_3$ and Ins(1,3,4,5)P $_4$ and the EC $_{50}$ value found for the release of Ca $^{2+}$ were much higher than the values found for the Ca $^{2+}$ -influx and the maximal rise in [Ca $^{2+}$] $_i$. These data suggest that some cellular component, not directly related to activation of phospholipase C, plays a role in the UTP activated Ca $^{2+}$ -entry process.

Stimulation of nucleotide receptors results in a decline in the prestimulated cyclic AMP level (Sipma *et al.*, 1994). Simultaneous activation of phosphoinositide hydrolysis and inhibition of adenylyl cyclase activity upon stimulation of P $_2$ -purinoceptors was observed in several tissues including rat hepatocytes (Okajima *et al.*, 1987) and mouse ventricular myocytes (Yamada *et al.*, 1992). The lack of effect of cyclic AMP on the nucleotide receptor-mediated phospholipase C pathway in DDT $_1$ MF-2 cells is not due to a compensatory decrease in cyclic AMP caused by nucleotide receptor stimulation because: (1) stimulation of cells with micromolar concentrations of UTP does not significantly reduce cyclic AMP, while at these concentrations UTP is able to raise [Ca $^{2+}$] $_i$, which is not affected by forskolin or isoprenaline pretreatment. (2) UTP decreases the isoprenaline-induced cyclic AMP content to about 2 times the level found with forskolin in the absence of UTP, while isoprenaline did not affect the UTP-induced increase in Ins(1,4,5)P $_3$, Ins(1,3,4,5)P $_4$ and [Ca $^{2+}$] $_i$. (3) Agonists of the nucleotide receptor decrease forskolin induced cyclic AMP to a level that is still sufficient

to reduce the histamine-induced increase in Ins(1,4,5)P $_3$ and Ins(1,3,4,5)P $_4$.

These observations suggest that, in contrast to P $_{2U}$ -purinoceptor stimulation, histamine H $_1$ -receptor stimulation is modulated by cyclic AMP-activated protein kinase A, which phosphorylates some component in the signal transduction pathway leading to activation of phospholipase C. Previous experiments showed that the P $_{2U}$ -receptor coupled G-protein that activates phospholipase C, is sensitive to pertussis toxin (Gerwins *et al.*, 1992; personal observation), in contrast to histamine H $_1$ -receptor coupled G-protein (White *et al.*, 1993; personal observation). These different G-proteins may function as a target for cyclic AMP discrimination. The cloning of the bovine and rat histamine H $_1$ -receptor (Fujimoto *et al.*, 1993; Yamashita *et al.*, 1991) has revealed the existence of phosphorylation sites in the third intracellular loop of the receptor, which is thought to interact with a putative G-protein. Such consensus sites for protein kinase A phosphorylation have not been found in the third intracellular loop of the recently cloned P $_{2U}$ -purinoceptor (Lustig *et al.*, 1993), suggesting another site for cyclic AMP discrimination.

In summary, the results show that cyclic AMP differentially affects histamine-induced Ins(1,4,5)P $_3$ and Ins(1,3,4,5)P $_4$ formation and that cyclic AMP regulates histamine H $_1$ receptor but not P $_{2U}$ -purinoceptor stimulation leading to the formation of inositol phosphates and a rise in [Ca $^{2+}$] $_i$.

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