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Desensitization of the histamine H_1-receptor and transcriptional down-regulation of histamine H_1-receptor gene expression in bovine tracheal smooth muscle

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1 We have investigated the role of protein kinase C (PKC) in the desensitization of histamine H_1-receptors and in the expression of the histamine H_1-receptor gene in airway smooth muscle.

2 Prolonged 4β-phorbol 12,13 dibutyrate (PDBu) pretreatment (4 h, 100 nM – 1 μM) of bovine trachealis caused a concentration-dependent loss of contraction in response to histamine H_1-receptor stimulation, which was associated with a concentration-dependent decrease in histamine-induced total [^{3}H]-inositol phosphates accumulation. In contrast, the responses to sodium fluoride, a direct G-protein activator, were unalterd by PDBu (100 – 300 nM) pre-incubation and only slightly reduced following incubation with 1 μM PDBu.

3 A selective PKC inhibitor, GF 109203X, partially blocked the PDBu (1 μM)-induced desensitization and completely blocked the effect of 100 nM PDBu, confirming the involvement of PKC.

4 Binding experiments using [^{3}H]-pyrilamine revealed a class of high-affinity binding sites within the range for the histamine H_1 receptor in airway smooth muscle. PDBu (1 μM) pretreatment for 4 h did not change the number of histamine H_1 receptors.

5 PDBu (1 μM) exposure caused a time-dependent reduction in the steady-state levels of histamine H_1-receptor mRNA, which was inhibited by pre-incubation with GF 109203X and by cycloheximide, a protein synthesis inhibitor.

6 Nuclear run-on assays revealed a 50% reduction in the rate of histamine H_1-receptor gene transcription after 17 h PDBu pretreatment, whereas mRNA stability was not affected by PDBu pretreatment (17 h).

7 In conclusion, we have shown a PKC-mediated desensitization of the histamine H_1-receptor in BTSM and a transcriptional down-regulation of the histamine H_1-receptor gene expression, which requires new protein synthesis.

Keywords: Histamine H_1-receptor; desensitization; airway smooth muscle; gene expression; protein kinase C

Introduction

The various biological actions of histamine, as a mediator in several (patho)physiological conditions and as a neurotransmitter in mammalian brain, are mediated through at least three histamine receptor subtypes (H_1, H_2 and H_3) of which the pharmacological, biochemical and molecular aspects were recently reviewed in an extensive manner (Hill, 1990; Arrang, 1994; Leurs *et al*., 1995). Although the histamine H_3-receptor has not yet been cloned, all three histamine receptors seem to belong to the superfamily of G-protein-coupled receptors. The histamine H_1-receptor is coupled, *via* a regulatory G_q-like protein, to phospholipase C, which upon activation mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in the generation of two secondary messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG) (Leurs *et al*., 1995). $InsP_3$ is responsible for the release of Ca^{2+} from intracellular stores whereas DAG is able to activate cytosolic protein kinase C (PKC), which seems to play a pivotal role in signal transduction (Nishizuka, 1989).

Several studies have highlighted the role of PKC in modulating the function of several G-protein coupled receptors and many have reported PKC-induced modulation of histamine H_1-receptor-mediated functional responses. For example, it has been demonstrated in isolated cell systems, like HeLa cells, DDT$_1$MF-2 cells, HUVE cells and GT1-7 cells, that acute activation of PKC, by tumour-promoting phorbol esters, can produce a marked inhibition of the histamine-induced inositol phosphate formation or a significant attenuation of intracellular Ca^{2+}-release upon histamine H_1-receptor activation, suggesting desensitization of the H_1-receptor (Smit *et al*., 1992; Dickenson & Hill, 1993; McCreath *et al*., 1994; Zamani *et al*., 1995). This phorbol ester-induced uncoupling of the H_1-receptor was antagonized by selective PKC-inhibitors, providing evidence that PKC was involved in the uncoupling of the histamine H_1-receptor, probably as a result of receptor phosphorylation. Recently, the genes encoding human, rat, mouse, guinea-pig and bovine histamine H_1 receptors have been cloned (Yamashita *et al*., 1991; De Backer *et al*., 1993; Fujimoto *et al*., 1993; Horio *et al*., 1993; Fukui *et al*., 1994; Traiffort *et al*., 1994; Inoue *et al*., 1996). The primary sequence of the histamine H_1-receptor protein revealed the existence of seven putative transmembrane domains with a characteristic large third cytoplasmic loop, which is thought to interact with a G-protein. This third cytoplasmic loop has several serine and threonine residues, which may serve as sites for phosphorylation by various protein kinases (Yamashita *et al*., 1991).

Although several studies have investigated the effects of PKC activation on histamine H_1-receptors at the protein level, no one has focused on the histamine H_1-receptor gene

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expression. In this study, we have investigated the effect of PKC activation, using 4β-phorbol 12,13-dibutyrate (PDBu), a direct PKC activator, on the regulation of the histamine H_1-receptor gene expression in bovine trachealis.

Methods

Tissue preparation and incubation

Bovine tracheas were obtained from a local abattoir and placed at room temperature in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.05. The tracheal smooth muscle was dissected free of epithelium and connective tissue and smooth muscle pieces (2×2 mm) were prepared in KH solution at room temperature. The bovine tracheal smooth muscle was exposed to one or more of the following agents : 4β-phorobol 12,13-dibutyrate (PDBu, 100 nM – 1 μM), 4α-PDBu (1 μM), GF109203X (1 μM), cycloheximide (10 μg ml^{-1}) and actinomycin D (5 μg ml^{-1}).

Contraction measurements

After a 4 h incubation period in the absence or presence of PDBu, the strips were washed and mounted in 10-ml organ baths containing oxygenated KH at 37°C. A resting tone of 2 g was applied. The tissues were allowed to equilibrate for 30 min, during which they were washed with fresh KH every 10 min and cumulative concentration-response curves were performed to histamine (10 μM – 10 mM) or sodium fluoride (NaF, 10 – 80 mM). Only one concentration-response curve was constructed for each tissue. Isometric contractile responses were measured using a Grass FT 0.3 force-displacement transducer and visualized on a computer screen using a commercially availllable software program (Codas; Dataq Instruments, Inc., OH, U.S.A.). The results were expressed as ratio of mg contraction to mg dry weight (24 h, 60°C).

Accumulation of total [^{3}H]-inositol phosphates

Accumulation of total [^{3}H]-inositol phosphates in slices of bovine trachealis was performed as described previously (Hall *et al*., 1990). In brief, slices (300×300 μm) of bovine trachealis were prepared using a McIlwain tissue chopper. Washed slices were incubated in a shaking water bath at 37°C for 30 min in oxygenated (95% O_2, 5% CO_2) KH solution and then incubated for a further 75 min in KH solution containing [^{3}H]-myo-inositol (30 μCi, final concentration 0.4 μM, total volume 8 ml) under the same conditions. The slices were then washed in KH solution and incubated in oxygenated KH solution with PDBu (1 μM, 300 nM or 100 nM for 4 h). The slices were finally washed in KH solution containing 5 mM lithium chloride (LiCl) and resuspended in 8 ml of medium. One hundred μl of the aliquots suspension were then transferred to microcentrifuge tubes containing 200 μl oxygenated KH solution with 5 mM LiCl. The slices were then stimulated with histamine (10 mM) or NaF (80 mM) for 20 min and the incubation was terminated by the addition of 100 μl ice-cold perchloric acid (10% w/v). Samples were neutralized with 750 μl KOH (0.15 M), centrifuged ($3000 \times g$, 10 min, 4°C) and 750 μl aliqouts of the supernatant was diluted to a final volume of 3 ml with Tris buffer (50 mM, pH 7.0). Total [^{3}H]-inositol phosphates were separated from free [^{3}H]-myo-inositol by anion-exchange chromatography (Hill & Kendall, 1987).

Measurement of the binding of [^{3}H]-pyrilamine

Bovine trachealis (control and PDBu-treated, 1 μM for 4 h) was homogenized using a Polytron in ice-cold 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5 containing 0.32 M sucrose, then centrifuged at $1000 \times g$ for 10 min to remove unhomogenized debris; the supernatant was centrifuged at $40{,}000 \times g$ for 20 min and the resulting pellet was washed and centrifuged again. The final pellet was resuspended in incubation buffer (50 mM Tris-HCl, pH 7.5 at 30°C), to give a final protein concentration of 3 – 5 mg ml^{-1} and was stored at 70°C until required for use. Protein was determined by the method of Lowry *et al*. (1951) with bovine serum albumin used as a standard.

Membranes (0.3 – 0.4 mg protein) were incubated in duplicate with various concentrations of [^{3}H]-pyrilamine (0.25 – 10 nM; specific activity 20 Ci mmol^{-1}, New England Nuclear) for 60 min at 30°C in a total volume of 500 μl. Nonspecific binding was determined in the presence of 10 μM triprolidine. Reactions were terminated by rapid filtration through Whatman GF/C glass fibre filters, presoaked in 0.3% (w/v) polyethylenimine for 5 h, using a Brandel cell harvester. The filters were washed 3 times with ice-cold buffer, transferred to scintillation vials containing 4.0 ml Filtron-X (National Diagnostics, Hull, U.K.) and counted in a liquid scintillation counter (Packard TRI-CARB 2200CA) at an efficiency of 36%. Data were analysed using a weighted nonlinear regression-computerized least-squares curve-fitting program (EBDA and LIGAND).

Northern blot analysis

Total RNA was isolated by phenol/chloroform extraction and isopropanol precipitation (Chomczynski & Sacchi, 1987). Poly (A)$^+$ RNA was prepared using the PolyA-Tract® mRNA system IV (Promega, Southampton, U.K.) according to the manufacturers instructions, subjected to 1% agarose/formaldehyde gels containing 20 mM morpholinosulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7) and blotted onto Hybond-N filters (Amersham, U.K.) by capillary blotting. A 1.2 kb (*Eco*RI/*Sac*I) fragment specific to the bovine histamine H_1-receptor cDNA and a 1.2 kb (*Pst*I/*Pst*I) fragment specific to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were labelled by a random primer labelling kit (Amersham) using [α-^{32}P]-dCTP (3000 Ci mmol^{-1}, Amersham). After prehybridization for 4 h at 42°C in a buffer containing 50% formamide, 4 × Standard Sodium Citrate (SSC), 50 mM Tris-HCl (pH 7.5), 5 × Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 250 μg ml^{-1} denatured salmon sperm DNA, the blots were hybridized overnight at 42°C with the labelled probes ($1 - 2 \times 10^6$ c.p.m. ml^{-1}). Following hybridization, the blots were washed to a high stringency (2 × SSC at 42°C, 1 × SSC at 42°C, 0.5 × SSC at 50°C and 0.1 × SSC at 55°C, all for 30 min. in the presence of 0.1% SDS) before exposure to X-OMAT-S film. After suitable exposure time (1 – 3 days), the autoradiographs were analyzed by a laser densitometer (PDI, NY, U.S.A.). The amount of histamine H_1-receptor mRNA was quantified relative to the amount of GAPDH on the same filter.

Nuclear run-on assay

Nuclei were prepared as described by Müller *et al.* (1993). Isolated nuclei were resuspended in Tris-HCl (10 mM, pH 7.4), $MgCl_2$ (5 mM), glycerol (50%), sorbitol (0.5 M), Ficoll (2.5%), spermidine (0.008%) and dithiothreitol (1 mM) and were stored at −70°C until use. Nuclei were incubated for 30 min at 27°C with 300 μCi [^{32}P]-uracil triphosphate, adenosine triphosphate (0.625 mM), cytosine triphosphate and guanosine triphosphate (0.31 mM), Tris-HCl (40 mM), NH_4Cl (150 mM), $MgCl_2$ (7.5 mM) and RNasin (120 u). DNA digestion was then carried out with a 15 min incubation at 27°C with RQ-1 DNase (75 u) in the presence of RNasin (40 u) before protein digestion for 3 h at 37°C with proteinase K (1 mg ml^{-1}) in buffer containing Tris-HCl (pH 7.4, 10 mM), EDTA (15 mM), SDS (3%) and heparin (3 mg ml^{-1}). RNA extraction was then carried out with a phenol, phenol/chloroform (1 : 1) and a chloroform wash and then precipitated three times with 100% ethanol in the presence of 1.33 M ammonium acetate. The radiolabelled RNA was dissolved in 100 μl Tris-EDTA buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and added to 2 ml hybridization solution (50% formamide, 5 × SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl pH 7.5, 5 × Denhardt's solution, 50 μg ml^{-1} yeast tRNA, 100 μg ml^{-1} salmon sperm DNA, 0.02 μg/ml poly (A^+) and 0.02 μg/ml poly (G^+). Hybridization was carried out at 42°C for 72 h to 10 μg of the immobilized plasmid pGEM3Z and to plasmids containing inserts of rat GAPDH cDNA and bovine histamine H_1-receptor cDNA as described previously under Northern blot analysis. The filters were first washed in buffer A (300 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1% SDS, 1 μg ml^{-1} Rnase A and 10 u ml^{-1} RNase T1) at 37°C for 30 min then in buffer B (10 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA and 0.4% SDS) to a stringency of 55°C for 30 min before autoradiography.

Drugs

The drugs used in these experiments were obtained from the following sources: histamine diphosphate salt, sodium fluoride, 4α- and 4β-phorbol 12,13-dibutyrate (PDBu), cycloheximide, actinomycin D, lithium chloride and Dowex 1X8-200 (chloride form) (Sigma Chemical Co, Bornem, Belgium), GF 109203X (3-[1-[3-dimethylamino) propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrolyl-2,5-dione) (Biomol, Sanver Tech, Boechout, Belgium). myo-[2-^{3}H]-inositol (18.0 Ci mmol^{-1}) and [α-^{32}P]-dCTP (3000 Ci mmol^{-1}) (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). [^{3}H]-pyrilamine (20 Ci mmol^{-1}, New England Nuclear) All drugs were dissolved in distilled water except for 4β-phorbol 12,13-dibutyrate (PDBu), 4α-PDBu and GF 109203X which were dissolved in DMSO. All control tissues were incubated with an equivalent amount of appropriate vehicle.

Data analysis

All data are expressed as mean ± s.e.mean. Tissue preparations from at least four different animals were used for each type of contraction experiment. Log EC_{50} values were determined in individual experiments and used to calculate mean values. Statistical analysis was carried out by unpaired Students *t*-tests or analysis of variance as appropriate, probability values of $P<0.05$ were considered significant.

Results

Effect of PDBu pretreatment on histamine-induced contractions and histamine induced ^{3}H-inositol phosphate accumulation

Histamine (100 nM – 1 μM) induced a concentration-dependent contraction of bovine trachealis with a log EC_{50} value of −4.05 ± −4.8 M ($n=7$). Exposure to PDBu (100 nM – 1 μM) for 4 h, caused a concentration-dependent inhibition to subsequent histamine concentration-response curve, with a reduction of the maximum response of 88 ± 6% ($n=5$) after 1 μM PDBu exposure (Figure 1A). These findings correlated with a concentration-dependent reduction in histamine-induced accumulation of total [^{3}H]-inositol phosphates in bovine trachealis slices following PDBu (100 nM – 1 μM for 4 h) exposure (Figure 1B).

Effect of GF 109203X upon desensitization of histamine-induced contractions

Incubation with GF 109203X (1 μM), a selective PKC inhibitor, 1 h prior to PDBu exposure, partially antagonized

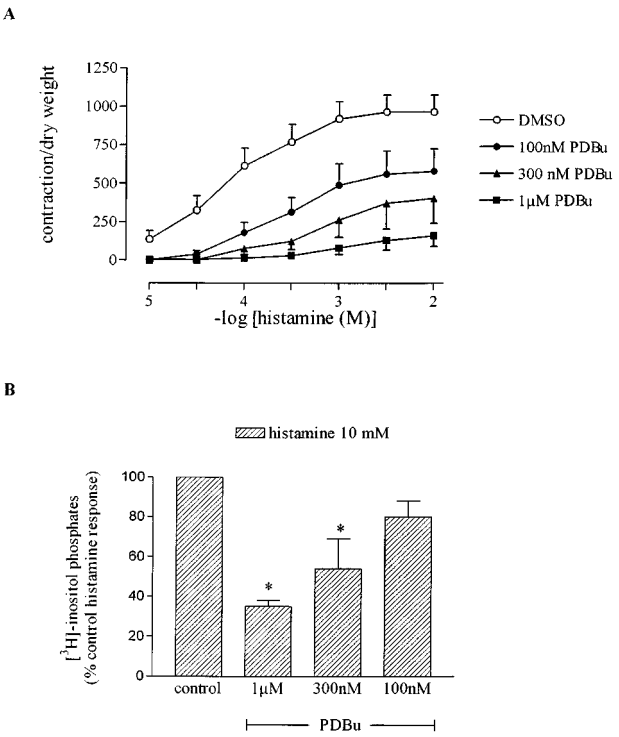

Figure 1 (A) Histamine-induced concentration-response curves in bovine trachealis after 4 h pretreatment with PDBu 1 μM ($P<0.001$ versus control), 300 nM ($P<0.001$ versus control), 100 nM ($P<0.05$ versus control) and DMSO (0.1%). Data shown are the mean ± s.e.mean of ⩾4 different experiments using preparations from ⩾4 different animals. Values are expressed as ratio of mg contraction to mg dry weight (24 h, 60°C). (B) Histamine (10 mM)-induced accumulation of [^{3}H]-inositol phosphates following 4 h pretreatment with different concentrations of PDBu. Bovine trachealis slices were prelabelled with [^{3}H]-myo-inositol for 75 min and pretreated with PDBu for 4 h. The slices were extensively washed and stimulated with histamine (10 mM) for 20 min in lithium chloride containing KH. The accumulated [^{3}H]-inositol phosphates were finally separated from free [^{3}H]-myo-inositol and counted as described under Methods. Values are the mean ± s.e.mean of 3 experiments and are expressed as % of [^{3}H]-inositol phosphates accummulation in control tissue stimulated with 10 mM histamine *$P<0.05$.

the inhibitory effect of 1 μM PDBu and completely reversed the effect of 100 nM PDBu pretreatment, while GF 109203X on its own had no effect on the histamine-induced contraction (Figure 2). Preincubation with 4α-PDBu (1 μM), a non-active phorbol ester, failed to modify the effects of histamine (results not shown).

Effect of PDBu-pretreatment on NaF responses

To investigate the possible site of the histamine H_1-receptor desensitization (at the level of the receptor or at a site distal in the signalling pathway), the contraction to sodium fluoride, a G-protein activator, was performed. NaF induced a concentration-dependent contraction of BTSM with a log EC_{50} value of -1.6 ± -2.5 ($n=4$). PDBu (300 – 100 nM) pretreatment (4 h) failed to alter significantly the NaF-induced contraction. Exposure to 1 μM PDBu caused a significant inhibition of the NaF-induced contraction, with a reduction of $45 \pm 4\%$ ($n=4$) of the maximum response (Figure 3A). This inhibition was, however, much smaller than the inhibition of the maximum response to histamine after 1 μM PDBu pretreatment ($45 \pm 4\%$ and $88 \pm 6\%$, respectively). These findings correlated with the accumulation of total [^{3}H]-inositol phosphates in bovine trachealis slices upon NaF stimulation, which were not altered following PDBu pre-incubation (100 nM – 1 μM, 4 h) (Figure 3B).

Receptor binding studies

Specific binding of [^{3}H]-pyrilamine accounted for 70 – 90% of the total binding. Analysis of the data revealed a class of high-affinity binding sites within the range for the histamine H_1 receptor in airway smooth muscle. The dissociation constant (K_d) and maximal binding capacity (B_{max}) of the H_1 receptor were not significantly different between vehicle- and PDBu-treated tissues after 4 h (K_d values of 1.28 ± 0.08 and 1.08 ± 0.13 nM, respectively; B_{max} values of 129.6 ± 6.1 and 142.9 ± 27.6 fmol mg^{-1} protein, respectively; $n=4$)

Northern blot analysis

Northern blot analysis of BTSM, revealed a band of 3.0 kb in size, corresponding to a histamine H_1-receptor mRNA, in agreement with findings of Yamashita *et al*., (1991) (Figure 4). Stimulation with PDBu (1 μM) for 4 h had no effect whereas

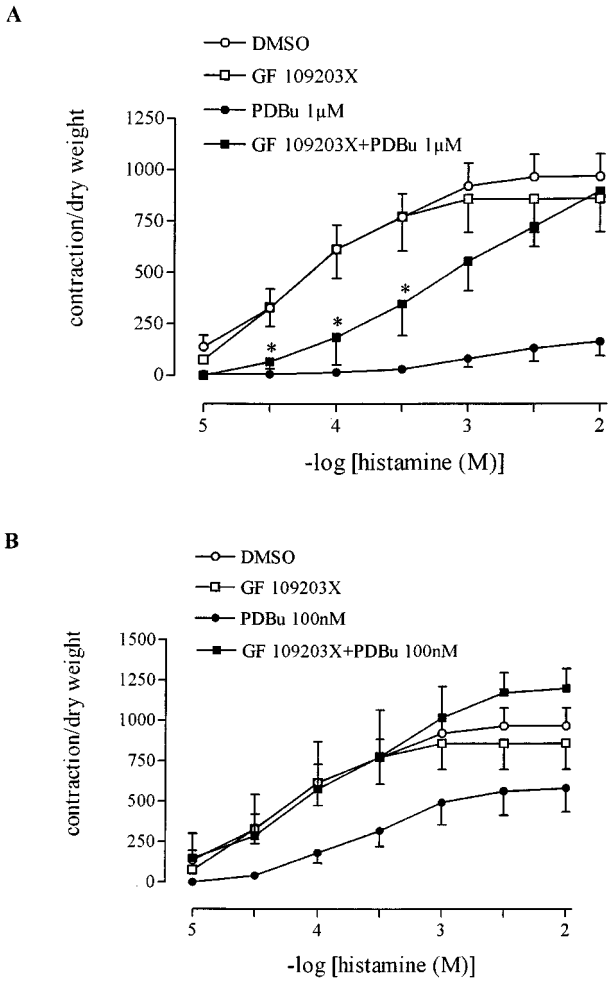

Figure 2 The effect of GF 109203X (1 μM) upon the histamine H_1-receptor desensitization induced by a 4 h exposure of bovine trachealis to 1 μM (A) and 100 nM (B) PDBu. The strips were exposed to PDBu in the presence or absence of GF 109203X (1 μM, added 1 h prior to PDBu) then extensively washed before concentration-response curves to histamine (10 μM – 10 nM) were performed. The effect of GF 109203X + PDBu was compared with PDBu alone, while GF 109203X and DMSO (0.1%) served as control. Data shown are the mean $\pm$ s.e.mean of 4 different experiments, using preparations from 4 different animals, $*P<0.05$.

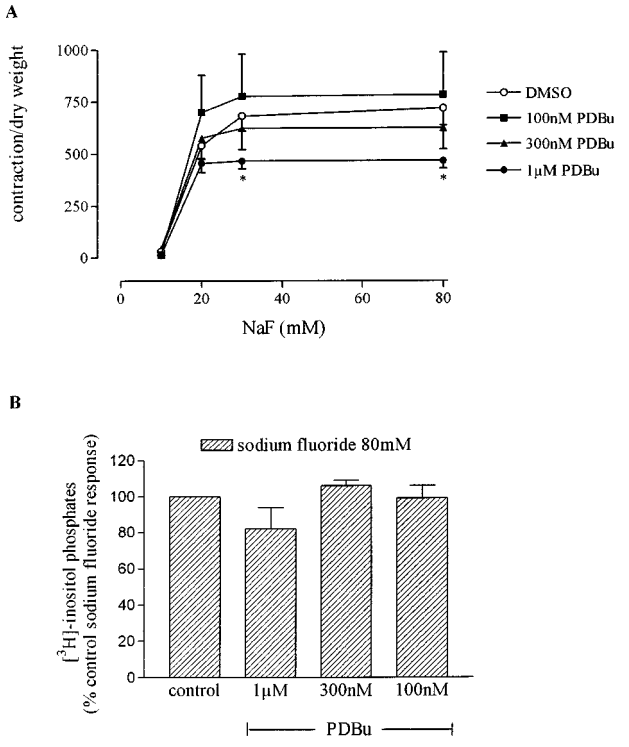

Figure 3 (A) Concentration-response curves to sodium fluoride in bovine trachealis following 4 h pretreatment with a range of concentrations of PDBu or DMSO (0.1%). Data shown are the mean $\pm$ s.e.mean of 4 different experiments, using preparations from 4 different animals, $*P<0.05$. (B) Sodium fluoride (80 mM) induced accumulation of [^{3}H]-inositol phosphates following 4 h pretreatment with different concentrations of PDBu. Bovine trachealis slices were prelabelled with [^{3}H]-myo-inositol for 75 min and pretreated with PDBu for 4 h. The slices were extensively washed and stimulated with sodium fluoride (80 mM) for 20 min in lithium chloride containing KH. The accumulated [^{3}H]-inositol phosphates were finally separated from free [^{3}H]-myo-inositol and counted as described under Methods. Values are the mean $\pm$ s.e.mean of 3 experiments and are expressed as % of [^{3}H]-inositol phosphates accummulation in control tissue stimulated with 10 mM histamine $*P<0.05$.

prolonged PDBu pretreatment (1 μM, 17 and 20 h) resulted in a down-regulation of histamine H_1-receptor mRNA steady-state level with a decrease of 22% at 17 h and 40% at 20 h (Figure 4). 4-α PDBu, the inactive form of the phorbol ester, had no effect on histamine H_1-receptor mRNA steady-state level (Figure 5A). Preincubation with GF 109203X completely inhibited the PDBu-induced decrease in mRNA steady-state level at 17 h (Figure 5A). To determine the mechanism of the PDBu-induced histamine H_1-receptor mRNA down-regulation, experiments were performed using cycloheximide, a protein synthesis inhibitor. Cycloheximide (10 μg ml^{-1}) produced superinduction (150%) of histamine H_1-receptor mRNA at 17 h. However, there was no difference in the histamine H_1-receptor mRNA level between incubations with cycloheximide in the presence or in the absence of PDBu for 17 h (Figure 5B).

To investigate whether PKC stimulation decreased histamine H_1-receptor mRNA expression by increasing mRNA degradation, the RNA polymerase inhibitor actinomycin D (5 μg ml^{-1}) was added to treated and untreated BTSM. The degradation rate of the histamine H_1-receptor mRNA was not significantly affected by 17 h PDBu treatment ($t_{1/2}$ of 4.5 h and 5 h for control and PDBu-treated tissues respectively) (Figure 6). To investigate whether the rate of histamine H_1-receptor

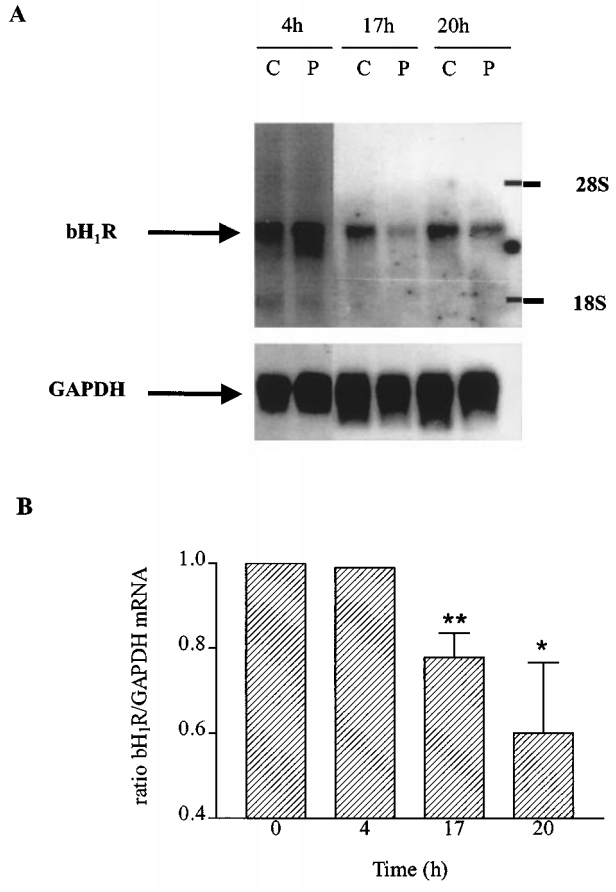

Figure 4 Northern blot analysis was performed with labelled cDNA probes for bovine histamine H_1-receptor (bH_1R) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with poly(A)$^+$RNA, as described under Methods. (A) Shows a representative Northern blot following DMSO (0.1%) and PDBu (1 μM) pretreatment for the times indicated (C = control, P = PDBu). (B) Shows bovine histamine H_1-receptor (bH_1R) mRNA levels relative to GAPDH mRNA after 4, 17 and 20 h preincubation with PDBu (1 μM) as assessed by laser densitometry. Data shown are the mean ± s.e.mean of 4 different experiments. $^*P<0.05$, $^{**}P<0.01$.

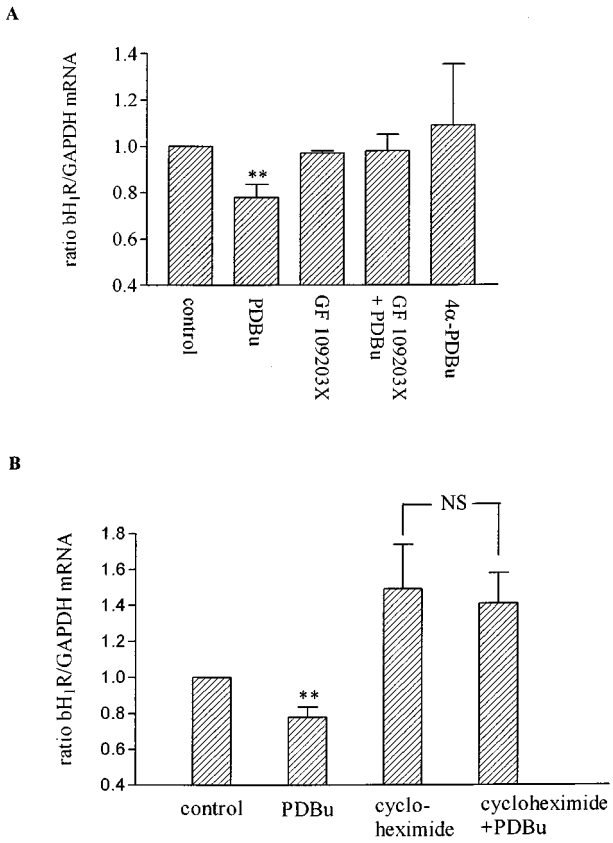

Figure 5 (A) Effect of 17 h incubation with PDBu (1 μM), GF 109203X (1 μM), a specific PKC antagonist and of the inactive phorbol ester 4α-PDBu (1 μM) on the steady-state level of bovine histamine H_1-receptor (bH_1R) mRNA in bovine trachealis as assessed by Northern blot analysis as described under Methods. GF 109203X was added 1 h prior to PDBu. Data represent the mean + s.e.mean of at least 3 different experiments, $^{**}P<0.01$. (B) Effect of cycloheximide (10 μg ml^{-1}), a protein synthesis inhibitor, on the steady-state level of bovine histamine H_1-receptor (bH_1R) mRNA in bovine trachealis following 17 h exposure time to PDBu (1 μM) as assessed by Northern blot analysis. Data represent the mean ± s.e.mean of 4 different experiments, $^{**}P<0.01$.

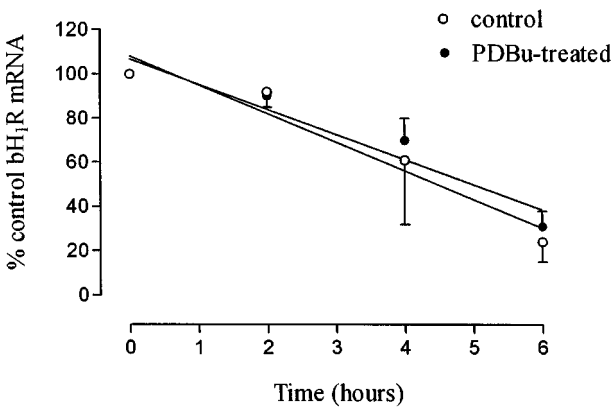

Figure 6 Untreated (DMSO, 0.1%) and PDBu-treated (1 μM for 17 h) bovine trachealis was incubated with actinomycin D (5 μg ml^{-1}) for the times indicated, after which Northern blot analyses were performed on poly(A)$^+$ RNA. The degradation rate of bovine histamine H_1-receptor (bH_1R) mRNA following PDBu pretreatment was compared to control tissue. Values shown are the mean + or − s.e.mean of 3 separate experiments.

gene transcription was changed following PDBu exposure, we performed nuclear run-on assay from isolated nuclei of control and PDBu-treated BTSM. As shown in Figure 7, the rate of transcription of newly-synthesized histamine H_1-receptor mRNA was reduced by 50% after 17 h PDBu-treatment.

Discussion

In this study we have investigated the effect of long-term phorbol ester treatment on histamine H_1-receptor responsiveness and on histamine H_1-receptor gene expression in BTSM. We have shown that PDBu caused a concentration-dependent inhibition of contraction of BTSM after 4 h pretreatment, and a time-dependent reduction in histamine H_1-receptor mRNA steady state-level.

Pre-incubation for 4 h with PDBu caused a significant loss of histamine-induced contraction. A PKC mediated mechanism is probably involved in this desensitization of the histamine H_1-receptor, since the PKC inhibitor GF 109203X antagonized the effect of PDBu whereas the inactive phorbol ester had no such effect. A small reduction in NaF-induced contractions was produced by 1 μM PDBu although lower concentrations were without effect. NaF has been demonstrated to interact directly with the Gq-protein to induce inositol phosphate formation in smooth muscle cells (Hall *et al*., 1990), thus bypassing the receptor-mediated process of the signal transduction pathway. Collectively, the data suggest that the desensitization following PDBu pretreatment at 100 and 300 nM is mediated at the level of the histamine H_1-receptor or at the coupling with its G-protein, since NaF contractions were unaffected. In contrast, NaF-induced contractions were slightly affected by the higher concentration of PDBu, suggesting an effect on the G-protein itself or at a site distal to the G-protein. Several sites of interaction of PKC distal to the receptor have been suggested, for example, inhibition of K^+ channels and reduction of extracellular calcium influx (Peppelenbosch *et al*., 1991), inhibition of phospholipase C activity (Cost *et al*., 1991) and activation of adenylate cyclase to produce cyclic AMP and subsequent activation of cyclic AMP-dependent PKA, which may inhibit GTP-mediated PIP_2 hydrolysis (Tachado *et al*., 1993). However, the site at which PKC, activated by higher concentrations of PDBu in our study, interacts in the cellular events remains speculative. We have also shown that the PDBu-induced loss of contractile responses to histamine was associated with a desensitization of the histamine H_1-receptor-mediated accumulation of total inositol phosphates. Moreover, PDBu pretreatment failed to modify the sodium fluoride-induced total inositol phosphates accumulation, confirming that the PDBu-induced desensitization of the histamine H_1-receptor occurred at the level of the G-protein or at the coupling with its G-protein.

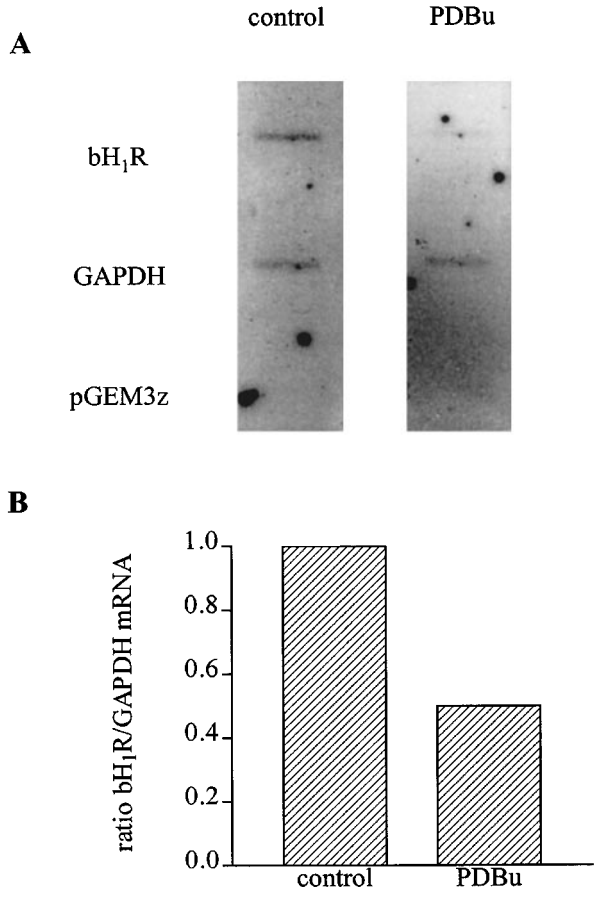

Figure 7 ^{32}P-labelled mRNA was transcribed *in vitro* from isolated nuclei obtained from untreated and PDBu-treated (17 h) bovine trachealis and hybridized to plasmid cDNAs immobilized on nylon membranes. The plasmids used were pGEM3Z, as a negative control and plasmids containing bovine histamine H_1-receptor (bH_1R) and GAPDH cDNA inserts. The data represent bovine histamine H_1-receptor (bH_1R) mRNA relative to GAPDH mRNA after densitometric measurement.

Desensitization of G-protein coupled receptors often involves phosphorylation by protein kinases, which appears to be a common mechanism of receptor regulation. Since PDBu-induced histamine H_1-receptor desensitization could be blocked by a selective PKC antagonist and since putative phosphorylation sites in the third cytoplasmic loop of the histamine H_1-receptor have been found (Yamashita *et al*., 1991), it could be hypothesized that the desensitization of histamine H_1-receptor is a result of a PKC-induced phosphorylation and hence uncoupling of the receptor. On the other hand, a decrease in histamine H_1-receptor number has recently been put forward as an explanation for dexamethasone-induced inhibition of inositol phosphate formation and intracellular calcium response to histamine H_1-receptor stimulation in airway smooth muscle cells, although no direct evidence (receptor binding experiments) was given (Hardy *et al*., 1996). In our study, however, PDBu pretreatment for 4 h induced neither a reduction in histamine H_1 receptor number nor a down-regulation of the histamine H_1-receptor mRNA steady-state level, providing more evidence that the observed desensitization is a result of receptor uncoupling, without a reduction in the number of receptors, at least following an incubation period of 4 h.

So far, a growing number of genes has been found to be regulated by tumour-promoting phorbol esters through a direct activation of PKC (Rahmsdorf & Herrlich, 1990). For example, muscarinic M_2 receptors are down-regulated by phorbol esters *via* an inhibitory effect on gene transcription (Rousell *et al*., 1995). In the present study we showed a significant time-dependent down-regulation of the histamine H_1-receptor mRNA steady-state level in bovine trachealis following PDBu exposure at 17 and 20 h. This phorbol ester-induced reduction in the histamine H_1-receptor mRNA level was completely antagonized by co-incubation with GF 109203X, a selective PKC inhibitor, suggesting a PKC-mediated effect, which was also confirmed by the lack of effect of 4α-PDBu (an inactive form of the phorbol ester). At the moment, however, it is not clear whether this reduction in the histamine H_1 receptor mRNA level is of any physiological

significance, since there is already an almost complete loss of the functional response to histamine within 4 h incubation with PDBu.

Using cycloheximide, a protein synthesis inhibitor, we found a marked accumulation of the histamine H_1-receptor mRNA in bovine trachealis. The underlying process, known as superinduction, is thought to result from to the loss of labile transcriptional repressors and mRNA-degrading enzymes, and is usually regarded as a direct consequence of the inhibition of protein synthesis (Mahadevan & Edwards, 1991). Alternatively, some protein synthesis inhibitors may act positively to activate transcription through an intrinsic ability to interact with molecules involved in intracellular signalling as has been shown, for example, for the induction of c-*fos* and c-*jun* in C3H 10T1/2 cells (Mahadevan *et al*., 1990). However, in our study, we did not investigate the exact mechanism by which cycloheximide causes superinduction of histamine H_1-receptor gene. Nevertheless, in the presence of cycloheximide, PDBu caused no alteration in mRNA level in bovine trachealis compared to incubation with cycloheximide alone, providing evidence that, subsequent to PKC activation, synthesis of at least one new protein is required to induce transcriptional down-regulation of histamine H_1-receptor mRNA. It has been shown that PKC activation phosphorylates and induces DNA binding activity of a number of proteins, including transcription factors like AP-1 and NF-κB, which in turn may alter transcription of other genes (Chiu *et al*., 1987; Rahmsdorf & Herrlich, 1990; Henkel *et al*., 1993). However, so far, the nature of the protein(s) induced by PKC activation in bovine trachealis is unknown.

Finally, experiments were performed to determine if the reduction in histamine H_1-receptor mRNA level in bovine trachealis, induced by activation of PKC, was a consequence of alterations in mRNA stability or was due to changes in the rate of gene transcription. After 17 h incubation with PDBu, no change in mRNA stability was found, suggesting that changes in mRNA stability did not appear to be responsible for the reduction in histamine H_1 receptor mRNA. Therefore, a decrease in transcription rate was suggested, which was confirmed by nuclear run-on assay which showed a 50% reduction in histamine H_1-receptor gene transcription, indicating that the histamine H_1-receptor gene is subject to transcriptional down-regulation following PKC activation.

In conclusion, we have shown in bovine trachealis that initially PDBu exposure (4 h) resulted in a PKC-mediated desensitization of the histamine H_1-receptor, which was followed (at 17 and 20 h) by transcriptional down-regulation of histamine H_1-receptor gene expression.

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References

ARRANG, J.M. (1994). Pharmacological properties of histamine receptor subtypes. *Cell. Mol. Biol.,* **40,** 275–281.

CHIU, R., IMAGAWA, M., IMBRA, R.J., BOCKOVEN, J.R. & KARIN, M. (1987). Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. *Nature,* **329,** 648–651.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

COST, H., BARREAU, P., BASSET, M., LE PEUCH, C. & GENY, B. (1991). Phorbol myristate acetate inhibits phosphoinositol lipid-specific phospholipase C activity via protein kinase C activation in conditions inducing differentiation in HL-60 cells. *Cell. Biochem. Funct.,* **9,** 263–273.

DE BACKER, M.D., GOMMEREN, W., MOEREELS, H., NOBELS, G., VAN GOMPEL, P., LEYSEN, J.E. & LUYTEN, W.H. (1993). Genomic cloning, heterologous expression and pharmacological characterization of a human histamine H1 receptor. *Biochem. Biophys. Res. Commun.,* **197,** 1601–1608.

DICKENSON, J.M. & HILL, S.J. (1993). Homologous and heterologous desensitization of histamine H1- and ATP-receptors in the smooth muscle cell line, DDT1MF-2: the role of protein kinase C. *Br. J. Pharmacol.,* **110,** 1449–1456.

FUJIMOTO, K., HORIO, Y., SUGAMA, K., ITO, S., LIU, Y.Q. & FUKUI, H. (1993). Genomic cloning of the rat histamine H1 receptor. *Biochem. Biophys. Res. Commun.,* **190,** 294–301.

FUKUI, H., FUJIMOTO, K., MIZUGUCHI, H., SAKAMOTO, K., HORIO, Y., TAKAI, S., YAMADA, K. & ITO, S. (1994). Molecular cloning of the human histamine H1 receptor gene. *Biochem. Biophys. Res. Commun.,* **201,** 894–901.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1990). Modulation of fluoroaluminate-induced inositol phosphate formation by increases in tissue cyclic AMP content in bovine tracheal smooth muscle. *Br. J. Pharmacol.,* **100,** 646–650.

HARDY, E., FARAHANI, M. & HALL, I.P. (1996). Regulation of histamine H$\sim$1 receptor coupling by dexamethasone in human cultured airway smooth muscle. *Br. J. Pharmacol.,* **118,** 1079–1084.

HENKEL, T., MACHLEIDT, T., ALKALAY, I., KRONKE, M., BENNERIAH, Y. & BAEUERLE, P.A. (1993). Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature,* **365,** 182–185.

HILL, S.J. (1990). Distribution, properties, and functional characteristics of three classes of histamine receptor. *Pharmacol. Rev.,* **42,** 45–83.

HILL, S.J. & KENDALL, D.A. (1987). Studies on the adenosine-receptor mediating the augmentation of histamine-induced inositol phospholipid hydrolysis in guinea-pig cerebral cortex. *Br. J. Pharmacol.,* **91,** 661–669.

HORIO, Y., MORI, Y., HIGUCHI, I., FUJIMOTO, K., ITO, S. & FUKUI, H. (1993). Molecular cloning of the guinea-pig histamine H1 receptor gene. *J. Biochem.,* **114,** 408–414.

INOUE, I., TANIUCHI, I., KITAMURA, D., JENKINS, N.A., GILBERT, D.J., COPELAND, N.G. & WATANABE, T. (1996). Characteristics of the mouse genomic histamine H1 receptor gene. *Genomics,* **36,** 178–181.

LEURS, R., SMIT, M.J. & TIMMERMAN, H. (1995). Molecular pharmacological aspects of histamine receptors. *Pharmacol. Ther.,* **66,** 413–463.

LOWRY, O.H., ROSEBOROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MAHADEVAN, L.C., WILLS, A.J., HIRST, E.A., RATHJEN, P.D. & HEATH, J.K. (1990). 2-Aminopurine abolishes EGF- and TPA-stimulated pp33 phosphorylation and c-fos induction without affecting the activation of protein kinase C. *Oncogene,* **5,** 327–335.

MAHADEVAN, L.C. & EDWARDS, D.R. (1991). Signalling and superinduction. *Nature,* **349,** 747–748.

MCCREATH, G., HALL, I.P. & HILL, S.J. (1994). Agonist-induced desensitization of histamine H1 receptor-mediated inositol phospholipid hydrolysis in human umbilical vein endothelial cells. *Br. J. Pharmacol.,* **113,** 823–830.

MULLER, F.U., BOHELER, K.R., ESCHENHAGEN, T., SCHMITZ, W. & SCHOLZ, H. (1993). Isoprenaline stimulates gene transcription of the inhibitory G protein alpha-subunit Gi alpha-2 in rat heart. *Circ. Res.,* **72,** 696–700.

NISHIZUKA, Y. (1989). The family of protein kinase C for signal transduction. *JAMA,* **262,** 1826–1833.

PEPPELENBOSCH, M.P., TERTOOLEN, L.G. & DE LAAT, S.W. (1991). Epidermal growth factor-activated calcium and potassium channels. *J. Biol. Chem.,* **266,** 19938–19944.

RAHMSDORF, H.J. & HERRLICH, P. (1990). Regulation of gene expression by tumor promoters. *Pharmacol. Ther.,* **48,** 157–188.

ROUSELL, J., HADDAD, E.B., MAK, J.C. & BARNES, P.J. (1995). Transcriptional down-regulation of m2 muscarinic receptor gene expression in human embryonic lung (HEL 299) cells by protein kinase C. *J. Biol. Chem.,* **270,** 7213–7218.

SMIT, M.J., BLOEMERS, S.M., LEURS, R., TERTOOLEN, L.G., BAST, A., DE LAAT, S.W. & TIMMERMAN, H. (1992). Short-term desensitization of the histamine H1 receptor in human HeLa cells: involvement of protein kinase C dependent and independent pathways. *Br. J. Pharmacol.,* **107,** 448–455.

TACHADO, S.D., ZHANG, Y. & ABDEL-LATIF, A.A. (1993). Protein kinase C is involved in cyclic adenosine monophosphate formation due to $PGF_{2\alpha}$ desensitization in bovine iris sphincter. *Invest. Ophtalmol. Vis. Sci.,* **34,** 2023–2032.

TRAIFFORT, E., LEURS, R., ARRANG, J.M., TARDIVEL-LACOMBE, J., DIAZ, J., SCHWARTZ, J.C. & RUAT, M. (1994). Guinea pig histamine H1 receptor. I. Gene cloning, characterization, and tissue expression revealed by in situ hybridization. *J. Neurochem.,* **62,** 507–518.

YAMASHITA, M., FUKUI, H., SUGAMA, K., HORIO, Y., ITO, S., MIZUGUCHI, H. & WADA, H. (1991). Expression cloning of a cDNA encoding the bovine histamine H1 receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 11515–11519.

ZAMANI, M.R., DUPERE, J.R. & BRISTOW, D.R. (1995). Receptor-mediated desensitisation of histamine H1 receptor-stimulated inositol phosphate production and calcium mobilisation in GT1-7 neuronal cells is independent of protein kinase C. *J. Neurochem.,* **65,** 160–169.

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