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Dual effects of histamine and substance P on intracellular calcium levels in human U373 MG astrocytoma cells: role of protein kinase C

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1 In human U373 MG astrocytoma cells agonist-induced increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are rapidly returned towards prestimulated levels. Examination of the effect of histamine and substance P on $[Ca^{2+}]_i$ in thapsigargin-treated cells has allowed a mechanism contributing to this effect to be characterized.

2 Histamine and substance P stimulated [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation in U373 MG cells. Concentration-response curves of [^{3}H]-IP_1 accumulation in suspensions of U373 MG cells in HEPES buffer containing 30 mM Li^+ yielded best-fit EC_{50} values of 19.1 ± 1.5 μM for histamine and 5.7 ± 1.3 nM for substance P.

3 In confluent monolayers of fura-2 loaded U373 MG cells perfusion with 100 μM histamine resulted in a transient 597 ± 50 nM increase in $[Ca^{2+}]_i$. The best-fit EC_{50} for histamine was 4.6 ± 2.2 μM. The initial, transient, histamine response was often followed by further small transient increases in $[Ca^{2+}]_i$.

4 Treatment of U373 MG cells with 5 μM thapsigargin, followed by the readdition of 1.8 mM Ca^{2+} to the perfusion buffer, resulted in a steady-state level of $[Ca^{2+}]_i$ 97 ± 5 nM above pretreated levels (measured 400 s after readdition of Ca^{2+}). Perfusion of histamine (100 μM, 100 s) caused a rapid decline in the thapsigargin-induced steady state level of $[Ca^{2+}]_i$. This effect of histamine was normally reversible upon washout. The best-fit EC_{50} for the histamine response was 0.8 ± 0.2 μM. Substance P (10 nM, 100 s) also caused a reduction in thapsigargin-induced steady-state levels of $[Ca^{2+}]_i$.

5 Neither 100 μM histamine nor 10 nM substance P inhibited the rate of quench of fura-2 fluorescence by Mn^{2+} in U373 MG cells pretreated with 5 μM thapsigargin, indicating that the depressant effect on steady-state raised $[Ca^{2+}]_i$ was probably not due to a block of Ca^{2+} entry.

6 The depressant effect of histamine on $[Ca^{2+}]_i$ was blocked by 1 μM mepyramine, and was partially reduced by pre-incubation with 1 μM staurosporine (61 ± 7% reduction) and with Ro 31-8220 (24 ± 10% and 50 ± 6% reduction by 1 and 10 μM Ro 31-8220, respectively). Pre-incubation with H-89 did not alter the depressant effect of histamine.

7 Neither 1 μM staurosporine nor 10 μM KN-62 inhibited the binding of [^{3}H]-mepyramine to guinea-pig cerebellar membranes, whereas it was reduced by 17 ± 1% and 55 ± 2% by 1 and 10 μM Ro 31-8220, respectively. However, [^{3}H]-IP_1 accumulation stimulated by histamine in U373 MG cells was not inhibited by 1 or 10 μM Ro 31-8220 and in 2 out of 3 experiments there was a significant potentiation of the response to histamine with both concentrations of Ro 31-8220. Staurosporine, 1 μM, similarly potentiated the response to 100 μM histamine in 3 out of 4 experiments. KN-62 (10 μM) did not stimulate histamine-induced [^{3}H]-IP_1 accumulation.

8 In HEPES buffer to which no Ca^{2+} had been added, histamine stimulated a transient 451 ± 107 nM increase in $[Ca^{2+}]_i$. Pretreatment with 1 μM and 10 μM Ro 31-8220 did not significantly alter the initial peak response to histamine, but slowed the rate at which histamine-induced increases in $[Ca^{2+}]_i$ were returned to prestimulated levels. Pretreatment with KN-62 had no significant effect on the response to histamine, but consistently inhibited the secondary slower phase of the decline in $[Ca^{2+}]_i$. H-89 did not alter the histamine response.

9 The effect of histamine in stimulating Ca^{2+} extrusion was not confined to U373 MG cells, since 100 μM histamine also caused a rapid decrease in steady-state levels of $[Ca^{2+}]_i$ in thapsigargin-treated human HeLa cells.

10 The results indicate that agonists which increase $[Ca^{2+}]_i$ via activation of phosphoinositide metabolism can also stimulate a homeostatic mechanism which acts to reduce $[Ca^{2+}]_i$. The balance of the evidence indicates that in U373 MG cells the latter effect most likely involves a PKC-mediated stimulation of a Ca^{2+}-extrusion pump.

Keywords: Histamine; substance P; intracellular calcium; protein kinase C; Ro 31-8220; U373 MG astrocytoma cells; Fura-2; calcium extrusion; inositol phosphates; thapsigargin;

Introduction

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is an important secondary messenger which in most cell types is maintained at concentrations around 20,000 fold lower than is found extracellularly (Clapham, 1995). Agonist-induced activation of a number of cell surface receptors, such as the histamine

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H_1-receptor, which are coupled through G-proteins to the β-form of phospholipase C (PLC-β), raise $[Ca^{2+}]_i$ by stimulating the hydrolysis of membrane bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the diffusable second messenger inositol 1,4,5-trisphosphate ($InsP_3$) (reviewed by Berridge, 1993). $InsP_3$ then acts, via specific receptors, on intracellular Ca^{2+} stores located in the endoplasmic reticulum to cause the rapid release of Ca^{2+} into the intracellular environment. The other product of phosphoinositide hydrolysis, diacylglycerol (DAG), remains in the lipid environment of the cell membrane and is responsible for the activation of protein kinase C (PKC) and, thus, can alter cellular processes via protein phosphorylation (Stabel & Parker, 1991; Nishizuka, 1992).

The phosphoinositide/Ca^{2+} signalling pathway has been the subject of numerous reviews (see e.g. Fisher *et al*., 1992; Berridge, 1993). Functionally it has been linked to various processes including long-term potentiation, immediate early gene expression and neurotransmitter release (reviewed in Simpson *et al*., 1995; Roche & Prentki, 1993; Henzi & MacDermott, 1992, respectively). In addition, a positive feedback effect of Ca^{2+} on PLC is necessary for the maintained stimulation of phosphoinositide hydrolysis (Wojcikiewicz *et al*., 1993). However, uncontrolled increases in $[Ca^{2+}]_i$ are potentially harmful to the cell and in order to maintain cell homeostasis, stimulated increases in $[Ca^{2+}]_i$ need to be rapidly returned towards resting levels. Regulation of $[Ca^{2+}]_i$ in the submicromolar range is predominantly under the control of a plasma membrane Ca^{2+} ATPase pump in most cells (Carafoli, 1994; Monteith & Roufogalis, 1995). This pump has a number of putative modulation sites, including a calmodulin binding domain and phosphorylation sites for protein kinase A (PKA) and PKC (Wang *et al*., 1992; Monteith & Roufogalis, 1995). Increases in Ca^{2+} extrusion via pump activation have been previously demonstrated in human platelets stimulated with thrombin (Rink & Sage, 1987) and in mouse pancreatic acinar cells stimulated with acetylcholine (Tepikin *et al*., 1992). It has also been shown that carbachol-induced activation of the plasma membrane Ca^{2+} pump in rat pancreatic acinar cells is independent of agonist-stimulated rises in $[Ca^{2+}]_i$ (Zhang *et al*., 1992), as is also apparent for the action of arginine-vasopressin in A7r5 smooth muscle cells (Byron & Taylor, 1995). The observation that the PKC-activator phorbol-12-myristate 13-acetate stimulates Ca^{2+} efflux in human erythrocytes (Wright *et al*., 1993) suggests the involvement of a protein kinase pathway and more recently evidence has been presented that PKC increases calcium pump activity in Jurkat T cells (Balasubramanyam & Gardner, 1995). However, the number of examples in which agonist-stimulated Ca^{2+} efflux has been shown to be sensitive to protein kinase inhibitors appears to be very limited.

In human U373 MG astrocytoma cells both histamine (Arias-Montaño *et al*., 1994) and substance P (Heuillet *et al*., 1993) stimulate phosphoinositide hydrolysis and mobilize Ca^{2+} from intracellular stores. However, in the course of a study of the characteristics of histamine-induced Ca^{2+} mobilization in U373 MG cells, we have observed that after pretreatment of cells with the endoplasmic Ca^{2+}-pump inhibitor thapsigargin (Takemura *et al*., 1989), addition of histamine resulted in a decrease in $[Ca^{2+}]_i$. We present here an investigation into this effect, together with some comparative observations on the action of substance P. A preliminary account of this work has been presented to the British Pharmacological Society (Young *et al*., 1996).

Methods

Accumulation of [^{3}H]-inositol phosphates in U373 MG cells

U373 MG cells (National Culture Collection, Porton Down) were grown to near confluence in Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F-12 (1:1 v/v; Gibco), containing 10% (v/v) foetal bovine serum and 2 mM glutamine (Gibco) and supplemented with penicillin (50 uml^{-1}), streptomycin (50 mg ml^{-1}) and amphotericin B (25 μg ml^{-1}) (Flow Laboratories) in flasks at 37°C in a CO_2-incubator (5% CO_2). The culture medium was removed and the cells washed in approximately 10 ml phosphate buffered saline (PBS) (in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5; pH 7.5) before addition of inositol-free DMEM containing 10% dialyzed calf serum (Gibco), 10 μM *myo*-inositol and 0.16 μM [^{3}H]-inositol (2.5 μCi ml^{-1}). The cells were then incubated for 20–24 h. The [^{3}H]-inositol-labelled cells were washed once with PBS/ethylenediaminetetracetic acid (EDTA) before dissociation with 10 ml trypsin/EDTA (500–750 BAEE units ml^{-1}/0.6 mM, Sigma). After centrifugation at 200 *g* for 3 min, the cells were resuspended in Krebs-Henseleit medium (in mM: NaCl 116, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 11; pH 7.4) which had been gassed with O_2/CO_2 (95:5 v/v), or in HEPES buffer (in mM: NaCl 120, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.6, HEPES 25 and D-glucose 11; pH 7.4) which had been gassed with 100% O_2. Cells (50 μl, approximately 1×10^5 cells) were added to microcentrifuge tubes containing 190 μl Krebs or HEPES, and LiCl (final concentration 30 mM), gassed and incubated for 15 min at 37°C before the addition of 10 μl agonist or distilled H_2O. Protein kinase inhibitors were also added at this stage, where appropriate. The tubes were again gassed and incubated for a further 30 min at 37°C. Incubations were terminated by the addition of 250 μl ice-cold 10% perchloric acid containing 1 mg ml^{-1} phytic acid and 1 mM EDTA. [^{3}H]-inositol phosphates were extracted by the addition of 0.4 ml tri-*n*-octylamine/1,1,2,-trichlorotrifluoroethane (1:1 v/v) to the samples, which were then mixed thoroughly before centrifugation at 950 *g* for 5 min. A portion (0.38 ml) of the upper, aqueous, phase was transferred to an insert vial, 3 ml 20 mM HEPES (pH 7.6) added, and the mixture applied to an AG 1-X8 (formate form, 100–200 mesh; Biorad) anion-exchange column.

[^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were eluted with 10 ml H_2O and 10 ml 60 mM ammonium formate/5 mM sodium tetraborate, respectively. [^{3}H]-inositol monophosphates ([^{3}H]-IP_1) were then eluted with 10 ml 200 mM ammonium formate/0.1 mM formic acid. Higher phosphates were eluted by sequential addition of 10 ml 400 mM ammonium formate/0.1 M formic acid (for [^{3}H]-inositol bisphosphates, [^{3}H]-IP_2) and 10 ml 800 mM ammonium formate/0.1 M formic acid (for [^{3}H]-inositol trisphosphates, [^{3}H]-IP_3). Quicksafe A (10 ml, Zinnser Analytic) was added to the eluant and the tritium content was determined by liquid scintillation counting. Within an experiment 4 replicate determinations were made of each incubation condition.

Dimethylsulphoxide (DMSO), used as the solvent for staurosporine, H-89 and KN-62 had no significant effect on either basal or histamine-stimulated [^{3}H]-IP_1 accumulation (99 ± 3% of control, $n = 3$) at a final concentration of 0.5% (v/v), the concentration present in Ca^{2+} measurements, or at 1%. However, a higher concentration, 4%, v/v, used only in the experiments in which the effect of staurosporine and KN-

62 on histamine-stimulated [^{3}H]-IP_1 accumulation was measured, consistently increased basal [^{3}H]-IP_1 accumulation, mean $23 \pm 8\%$ increase (statistically significant only when analysed by a paired t test over the 7 experiments) and inhibited the response to 100 μM histamine ($33 \pm 6\%$ inhibition). In all measurements with staurosporine, H-89 and KN-62 the equivalent concentration of DMSO was present in controls.

Cyclic AMP accumulation in U373 MG cells

U373 MG cells were harvested as above, resuspended in HEPES buffer containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 50 μl added to microcentrifuge tubes. After 15 min at 37°C, 50 μl of HEPES buffer containing 0.5 mM IBMX, and where appropriate, histamine or forskolin, was added, and the reaction left for 10 min at 37°C before termination with 100 μl 10% perchloric acid containing 1 mM EDTA. Cyclic AMP was extracted by use of a modified version of the trioctylamine/freon method described above. Briefly, samples were centrifuged at 2000 g for 5 min and 180 μl of the supernatant added to fresh microcentrifuge tubes; 0.4 ml of trioctylamine/1,1,2-trichlorotrifluoroethane (1:1 v/v) was added to the samples, which were mixed and centrifuged as above. The cyclic AMP content of a portion (50 μl) of the upper, aqueous phase was measured by a commercially available cyclic AMP ^{3}H-assay system (Amersham).

Fluorimetric analysis of calcium concentration in cells

U373 MG cells were grown to confluency on sterilized coverslips (9×22 mm, Chance Propper Ltd.) in culture dishes (35×10 mm, Bibby) in DMEM/F12 medium (see above for composition). Two coverslips were placed in each culture dish and where effects of protein kinase inhibitors were being examined the responses with and without inhibitor were compared using pairs of coverslips from the same culture dish. The coverslips were washed twice with HEPES buffer and then incubated in the dark for 1 h at room temperature, *circa* 22°C, in HEPES buffer containing 2 μM fura-2 acetoxymethyl ester (fura-2 AM) and 1 mg ml^{-1} bovine serum albumin. At the end of this time the coverslips were washed twice with HEPES buffer and then incubated for a further 30 min in the dark at room temperature. For experiments in which the effects of the protein kinase antagonists Ro 31-8220, H-89 and KN-62 were examined, U373 MG cells were seeded on coverslips as normal, then after 1 h, the medium was replaced with DMEM/F12 medium supplemented with 2 mM glutamine only, since agonist-induced decreases in intracellular Ca^{2+} appeared to be sensitive to the foetal bovine serum used to supplement the DMEM/F12 medium. The coverslips were then left for 20–24 h before being loaded with fura-2 AM as normal. HeLa cells were grown to confluency on coverslips as above, in DMEM (Gibco) containing 5% (v/v) foetal bovine serum and 5% (v/v) neonate calf serum (Gibco) and supplemented with penicillin (50 uml^{-1}) and streptomycin (50 mg ml^{-1}) (Flow Laboratories).

The coverslips were then mounted in cuvettes (4.5 ml, Kartell, u.v. grade) and continuously perfused at room temperature with HEPES buffer at a rate of 10 ml min^{-1}, with or without drugs, via a five-port valve (Omnifit). Fluorescence was monitored at an emission wavelength of 510 nm, after excitation at 340 and 380 nm, as described by Grynkiewicz *et al.* (1985), on either an Hitachi F-2000 spectrophotometer or a Shimadzu RF-5001PC spectrofluorophotometer. In experiments with the Hitachi F-2000 the approximate concentration of free intracellular calcium $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence at 340 and 380 nm (determined after subtraction of background autofluorescence at 340 and 380 nm remaining after the addition of 2 μM ionomycin + 5 mM $MnCl_2$ in Ca^{2+}–free HEPES at the end of each run) by means of a calibration curve constructed by the addition of 1 μM fura pentapotassium salt to a range of Ca^{2+}/ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) buffers (in mM: KCl 100, $MgCl_2$ 1, EGTA 10, HEPES 10 and $CaCl_2$ as appropriate), pH 7.2 at 22°C. Approximate $[Ca^{2+}]_i$ in the Shimadzu RF-5001PC was calculated from the equation (Grynkiewicz *et al.*, 1985): $[Ca^{2+}]_i = K_d \times \{(R - R_{min})/(R_{max} - R)\} \times \{F_{min}\ (380\ nm)/F_{max}\ (380\ nm)\}$ where K_d is the dissociation constant of Ca^{2+} for fura-2 (135 nM at 22°C), R_{min} and R_{max} are the maximal and minimal fluorescent ratios calculated from Ca^{2+}/EGTA buffers as above, and $F_{min}(380)$ and $F_{max}(380)$ are the fluorescent intensities, measured at 380 nm, in the absence and in the presence of saturating concentrations of Ca^{2+}, respectively.

In experiments designed to measure the rate of Mn^{2+} entry into cells loaded with fura-2 excitation was at 360 nM, the isobestic excitation wavelength for fura-2 $\pm$ Ca^{2+}, and emission at 510 nM. Under these conditions the decrease in fluorescent intensity (quench) reflects the extent to which intracellular fura-2 has been complexed by Mn^{2+}.

Measurement of [^{3}H]-mepyramine binding to guinea-pig cerebellar membranes

Membrane preparations from cerebella of Dunkin-Hartley strain guinea-pigs (males, 250–600 g; Tucks, Battlebridge, Essex) were prepared as described previously (Gibson *et al.*, 1993). Incubations in 10 mM Tris-HCl buffer, pH 7.5, contained 0.65 nM [^{3}H]-mepyramine, inhibitor and cerebellar membranes (0.16 mg protein) in a total volume of 1.00 ml (4 replicates at each inhibitor concentration, 8 replicates in the absence of inhibitor). Non-specific binding of [^{3}H]-mepyramine (17–31% of total binding) was determined as the binding insensitive to inhibition by 1 μM temelastine. Where inhibitors were dissolved in dimethyl sulphoxide (DMSO) equivalent amounts of DMSO (1%, v/v) were present throughout, although at this concentration DMSO had no significant effect on the receptor-specific binding of [^{3}H]-mepyramine. Equilibration was for 60 min at 30°C and was terminated by filtration through Whatman GF/B glass fibre paper, pre-soaked in 0.3% (w/v) polyethylenimine for 3–5 h, by use of a Brandel (Gaithersburg, Md, U.S.A.) cell harvester. The filters were washed with ice-cold buffer and then transferred to scintillation insert vials containing 4.0 ml scintillator (Emulsifier-Safe, Packard) and allowed to stand at room temperature for at least 2 h before determination of tritium by liquid scintillation counting.

Analysis of data

Concentration-response data for agonist-induced [^{3}H]-IP_1 accumulation and increases in $[Ca^{2+}]_i$ were fitted by non-linear regression to a Hill equation (logistic equation). The actual equation fitted was:

$$\text{Response} = \text{Resp}_{max}\ C^{n_H}/(C^{n_H} + EC_{50}{}^{n_H})$$

where $Resp_{max}$ is the maximum response, C is the agonist concentration, n_H is the Hill coefficient and EC_{50} is the

concentration giving the half-maximal response. Each point was weighted according to the reciprocal of its variance.

Statistical comparison of parameters characterizing two concentration-response curves was made by fitting the curves simultaneously (using the NAG library routine E04FDF) and assessing the increase in the residual sum of squares when parameters were constrained to be the same for both curves, by calculating the *F*-statistic (Rodbard, 1974):

$$F = \{(SS_2 - SS_1)/(df_2 - df_1)\}/(SS_1/df_1)$$

where SS_2 is the sum of squares when a parameter is shared (df_2 degrees of freedom) and SS_1 is the sum of squares when all parameters are allowed to float freely for each curve (df_1 degrees freedom).

The rate constant for the decline in fura-2 fluorescence measured at 510 nm after excitation at 360 nm following addition of Mn^{2+} was obtained by fitting (GraphPad Prism) a monoexponential function:

$$F = (F_0 - F_i).\exp(-k.t) + F_i$$

where k is the rate constant and F_0 and F_i are the fluorescent intensities at time zero and time infinity, respectively.

The statistical significance of differences between sample means was made by use of Student's *t* test or, where appropriate, paired *t* test. $P<0.05$ was considered significant. Where *post hoc* comparisons were made between multiple treatments within the same experiment, the significance of differences was assessed with Student-Newman-Keuls multiple range test.

Drugs

Myo-[2-^{3}H]-inositol, 18–24 Ci mmol^{-1}, was obtained from New England Nuclear, [*pyridinyl*-5-^{3}H]-mepyramine, 27 Ci mmol^{-1}, from Amersham International, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), fura-2 acetoxymethyl ester (fura-2 AM), fura-2 pentapotassium salt (fura-2), histamine dihydrochloride, L-histidine, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES), imidazole, 3-isobutyl-1-methylxanthine, mepyramine maleate, perchloric acid, phytic acid, thapsigargin and tri-*n*-octylamine from Sigma; 1,1,2-trichlorotrifluoroethane (freon) from Aldrich; H-89 (N-[(2)-*p*-bromocinnamylamino)ethyl)]-5-isoquinoline sulphonamide), KN-62 (I-[*N*, *O*,bis(5-isoquinolinesulphonyl-*N*-methyl-L-tyrosyl]-4-phenylpiperazine), Ro 31-8220 ([1-[3-(amidinothio)-propyl-1H-indoyl-3-yl)-3-(1-methyl-1H-indoyl-3-yl)-maleimide -methane sulphonate) and staurosporine from Calbiochem; thioperamide and [Sar^9, Met(O$_2$)^{11}]-substance P from RBI; substance P from Genosys. Ranitidine was a gift from the Glaxo Institute of Applied Pharmacology (Cambridge).

Results

Histamine- and substance P-stimulated [^{3}H]-IP accumulation in U373 MG cells

Histamine (100 μM) produced a 540±8% stimulation of basal [^{3}H]-IP$_1$ accumulation in U373 MG cells incubated in Krebs-Henseleit medium in the presence of 30 mM Li$^+$ after a 30 min incubation at 37°C (weighted mean±s.e.mean from 6 experiments; basal level of [^{3}H]-IP$_1$ 1727±13 d.p.m.). The accumulation of [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ was also stimulated, by 276±12 and 187±22%, respectively ($n=3$), but [^{3}H]-IP$_1$ was much the major fraction (83±2% of total [^{3}H]-IP$_1$+[^{3}H]-IP$_2$+[^{3}H]-IP$_3$, $n=3$). The best-fit EC_{50} for histamine-induced [^{3}H]-IP$_1$ production, 2.4±0.2 μM, was in good agreement with the value of 5.4±0.5 μM determined in an earlier study, in which it was shown that the response is H_1-receptor-mediated (Arias-Montaño *et al*., 1994). However, since determinations of intracellular Ca^{2+} levels in U373 MG cells were carried out in a HEPES buffer (see Methods for composition), measurements were also made in this medium (Figure 1). Basal [^{3}H]-IP$_1$ accumulation in HEPES medium was 1432±20 d.p.m. and was stimulated 389±12% by 100 μM histamine ($n=3$). The best-fit EC_{50} for histamine-induced [^{3}H]-IP$_1$ production in HEPES buffer was 19.1±1.5 μM.

Substance P (100 nM) also stimulated [^{3}H]-IP$_1$ accumulation in HEPES buffer, 349±11% of basal ($n=4$). The best-fit value of EC_{50} from a concentration-response curve constructed from the combined data from 4 independent experiments (Figure 1) was 5.7±1.3 nM. Substance P also stimulated [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ accumulation with EC_{50} values of 8.7±2.7 and 7.8±2.6 nM, respectively ($n=4$). These values compare with an EC_{50} of 3.4±0.4 nM for substance P-induced [^{3}H]-IP accumulation (95% of [^{3}H]-inositol phosphates coeluting with [^{3}H]-IP$_1$) obtained in U373 MG cells after incubation for 2 h in Krebs-Henseleit medium containing 1.2 mM $CaCl_2$ (Heuillet *et al*., 1993). The same study demonstrated that the action of substance P was via the NK_1-receptor (Heuillet *et al*., 1993).

Histamine-stimulated Ca^{2+} mobilization in U373 MG cells

The pattern of histamine-stimulated Ca^{2+} mobilization in U373 MG cells varied between coverslip cultures. Most

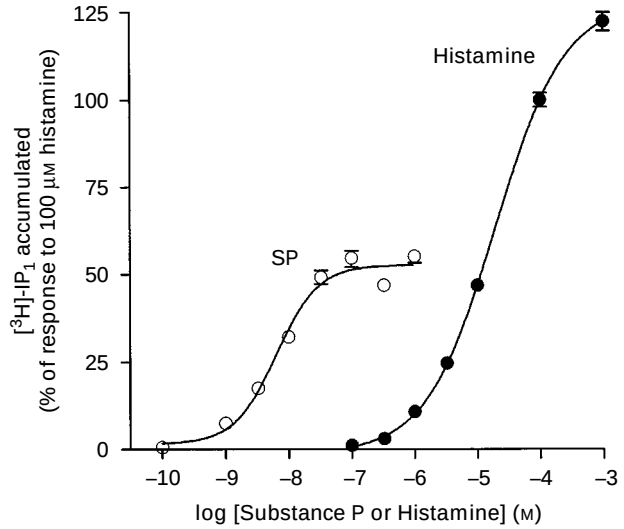

Figure 1 Stimulation of [^{3}H]-IP$_1$ accumulation by histamine and substance P (SP) in U373 MG cells. Values are expressed as a percentage of the response to 100 μM histamine, which was measured in every experiment. Basal [^{3}H]-IP$_1$ accumulation was subtracted before calculation of the agonist-stimulated response. Each point represents the weighted mean and s.e.mean (vertical lines) from 3 (histamine) or 4 (substance P) experiments. The curves drawn are the best-fit lines to the Hill equation (see Methods). Best-fit values of EC_{50} 5.7±1.3 nM and 19.1±1.5 μM for substance P and histamine, respectively (best-fit Hill coefficients 1.20±0.20 and 0.82±0.03; best-fit maximum responses 53±3 and 128±1% of the response to 100 μM histamine).

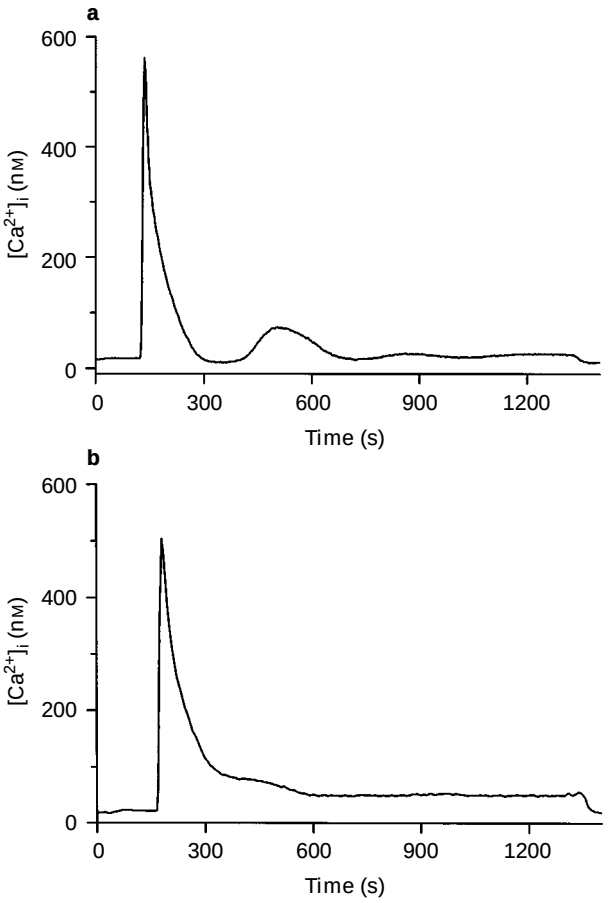

Figure 2 Histamine-stimulated increases in $[Ca^{2+}]_i$ in U373 MG cells. U373 MG cells, grown to confluency on cover-slips and preloaded with fura-2, were continuously perfused with HEPES buffer in the presence or absence of 100 μM histamine as described under Methods. (a) The trace is representative of 6 experiments in which histamine responses returned to prestimulated levels and were followed by further transient increases. Histamine was added at 100 s and replaced with medium alone at 1300 s. (b) The trace is representative of 7 experiments in which histamine responses did not return to prestimulated levels. Histamine was added at 150 s and replaced with medium alone at 1300 s.

commonly, histamine-induced increases in $[Ca^{2+}]_i$ consisted of an initial peak response which rapidly returned to pre-stimulated levels and was frequently followed by one or more small transient increases (Figure 2a). In these cells 100 μM histamine stimulated a 597 ± 50 nM increase in $[Ca^{2+}]_i$ ($n=6$). The mean resting $[Ca^{2+}]_i$ in U373 MG cells was 23 ± 2 nM ($n=23$). The EC_{50} for the initial peak increase was 4.6 ± 2.2 μM ($n=6$). The initial peak histamine response was not significantly altered when 1 mM EGTA was included in the perfusion buffer for 100 s before the addition of histamine ($17 \pm 9\%$ reduction, $n=3$). However, the secondary, transient increases in $[Ca^{2+}]_i$ were abolished by 1 mM EGTA. Incubation with 1 μM mepyramine for 100 s before the addition of histamine reduced the peak response to 100 μM histamine to a 27 ± 5 nM increase in $[Ca^{2+}]_i$.

The other major pattern of histamine-stimulated Ca^{2+} mobilization observed in the course of the study consisted of an initial transient peak response which did not completely return to prestimulated levels, but instead approached a plateau phase (Figure 2b). The peak increase in $[Ca^{2+}]_i$ of 447 ± 29 nM in response to 100 μM histamine ($n=7$) was not significantly less than that of the first response pattern, but $[Ca^{2+}]_i$ remained elevated during the secondary phase of the response, such that 300 s after the addition of 100 μM histamine the plateau level was 55 ± 11 nM above prestimulated levels ($n=7$).

Effect of histamine and substance P on $[Ca^{2+}]_i$ after emptying of intracellular stores with thapsigargin

Incubation with 5 μM thapsigargin in nominally Ca^{2+}-free HEPES (no added Ca^{2+}) caused a transient rise in $[Ca^{2+}]_i$ of 143 ± 6 nM above resting levels in U373 MG cells ($n=27$) (Figure 3a). Readdition of 1.8 mM Ca^{2+} to the perfusion buffer produced a secondary peak increase in $[Ca^{2+}]_i$ of 178 ± 8 nM, which declined to a slowly falling 'plateau' phase (97 ± 5 nM above resting levels, measured 400 s after the readdition of Ca^{2+}, $n=27$) (Figure 3a). Histamine (100 μM, 100 s application) caused a slow decline in the thapsigargin-induced plateau phase to increase from 0.010 ± 0.006 to 1.08 ± 0.09 nM s^{-1} (measured as the gradient of the initial portion of the histamine-induced decrease in $[Ca^{2+}]_i$, $n=3$) (Figure 3b). This effect on the initial rate of decline was concentration-dependent with a best-fit EC_{50} of 0.8 ± 0.2 μM and Hill coefficient of 0.82 ± 0.08 (Figure 4). The response to histamine was usually reversible on washout and a second 100 s application of 100 μM histamine, 400 s after washout of the initial application of 100 μM histamine, stimulated a 0.85 ± 0.02 nM s^{-1} decrease in $[Ca^{2+}]_i$ ($n=3$) (Figure 3b).

Perfusion of the H_1-receptor antagonist mepyramine (1 μM) for 130 s before the addition of histamine abolished the inhibitory effect on $[Ca^{2+}]_i$ by 100 μM histamine ($n=3$). The effects of antagonists were further examined by using the decrease in $[Ca^{2+}]_i$ induced by the first 100 s application of histamine as a control response, before perfusing with antagonist and then examining the effect of a second application of histamine, 400 or 500 s after the first, in the continued presence of antagonist. Mepyramine (1 μM) strongly inhibited the effect of the second application of 100 μM histamine (Figure 3d) (mean initial rate of decline 0.02 ± 0.02 nM s^{-1} after mepyramine, $n=3$), whereas 10 μM ranitidine and 1 μM thioperimide, H_2- and H_3- selective antagonists, respectively, were without significant effect ($n=3$). Similarly, neither 100 μM L-histidine nor 100 μM imidazole, controls for non-receptor effects of histamine, had a significant effect on the thapsigargin-induced plateau phase ($n=3$).

Substance P, 10 nM for 100 s, also caused a marked fall in $[Ca^{2+}]_i$ in U373 MG cells pretreated with thapsigargin (Figure 3c). However, the effect of substance P was not so readily reversed on washout as that of histamine (compare (b) and (c) in Figure 3). The effect of substance P was mimicked by the NK_1-selective agonist [Sar^9, $Met(O_2)^{11}$]-substance P (1 μM), consistent with the previous finding that the NK_1-receptor is the only subtype present in U373 MG cells (Heuillet *et al.*, 1993).

Effect of histamine and substance P on the rate of quench of fura-2 fluorescence by Mn^{2+} in U373 MG cells

The observed $[Ca^{2+}]_i$ in thapsigargin-treated cells is a steady-state balance between Ca^{2+} entry and Ca^{2+} extrusion across the plasma membrane. To determine whether the histamine-stimulated decrease in the thapsigargin-induced plateau phase of raised $[Ca^{2+}]_i$ was due to inhibition of Ca^{2+} entry, Ca^{2+} in the HEPES buffer was substituted with 750 μM Mn^{2+} and the rate of Mn^{2+} entry into U373 MG cells was measured by the ability of this ion to quench fura-2 fluorescence. Perfusion with 750 μM Mn^{2+} caused a time-dependent decrease in the level of

fura-2 fluorescence (measured at 510 nm after excitation at 360 nm) which approximated well to a monoexponential decay with a rate constant of $4.25 \pm 0.03 \times 10^{-3}$ s^{-1} ($n=6$) (Figure 5a). Pretreatment of the cells with 5 μM thapsigargin in 0 Ca^{2+} HEPES for 15 min increased the rate constant for Mn^{2+} quench to $5.36 \pm 0.02 \times 10^{-3}$ s^{-1} ($n=6$). The inclusion of 100 μM histamine in the perfusing buffer for 100 s before the addition of Mn^{2+} did not reduce the rate of Mn^{2+} quench in the thapsigargin-pretreated cells (rate constant for Mn^{2+} in the continued presence of histamine $5.60 \pm 0.02 \times 10^{-3}$ s^{-1}, $n=7$) (Figure 5a). Similarly, substance P did not reduce the rate of thapsigargin-stimulated Mn^{2+} quench of fura-2 fluorescence in U373 MG cells (Figure 5b). In this set of experiments, 250 μM Mn^{2+} was used to quench the fura-2 signal. A 15 min preincubation with 5 μM thapsigargin increased the rate of Mn^{2+} quench from $1.44 \pm 0.12 \times 10^{-3}$ s^{-1} to $4.71 \pm 0.71 \times 10^{-3}$ s^{-1}. When substance P (10 nM, 100 s before Mn^{2+}) was included in the perfusing buffer the rate of Mn^{2+} quench of fura-2 fluorescence after thapsigargin treatment was $5.02 \pm 0.35 \times 10^{-3}$ s^{-1} (Figure 5b).

Role of protein kinases in histamine-stimulated decreases in $[Ca^{2+}]_i$ in U373 MG cells

To examine whether the action of histamine on reducing $[Ca^{2+}]_i$ occurred via the activation of protein kinases, the effects of various protein kinase inhibitors were investigated. Preincubation of U373 MG cells with the relatively non-specific protein kinase inhibitor staurosporine (1 μM, for 15 min), before mounting the coverslips in the perfusion

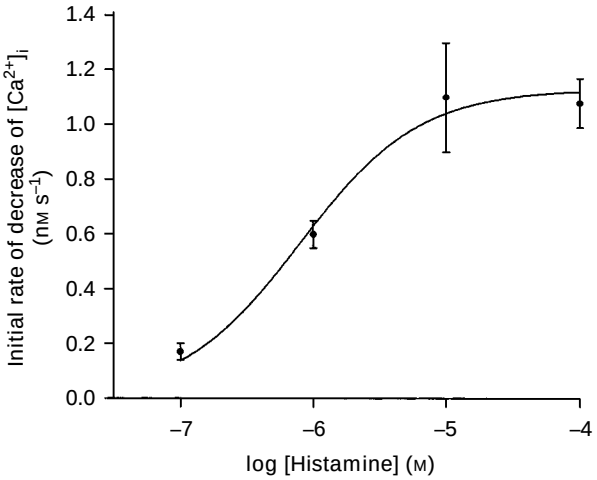

Figure 4 Concentration-response curve for the histamine-induced decrease in $[Ca^{2+}]_i$ in thapsigargin-treated U373 MG cells. The experimental protocol was as in Figure 3b. Each point represents the mean and s.e.mean (vertical lines) from 3 experiments in which the response to a 100 s application of histamine was measured as the initial gradient of the histamine-induced decrease in $[Ca^{2+}]_i$. The curve is a best-fit line to a Hill equation (see Methods). Best fit values: EC_{50} 0.8 ± 0.2 μM and Hill coefficient 0.82 ± 0.08.

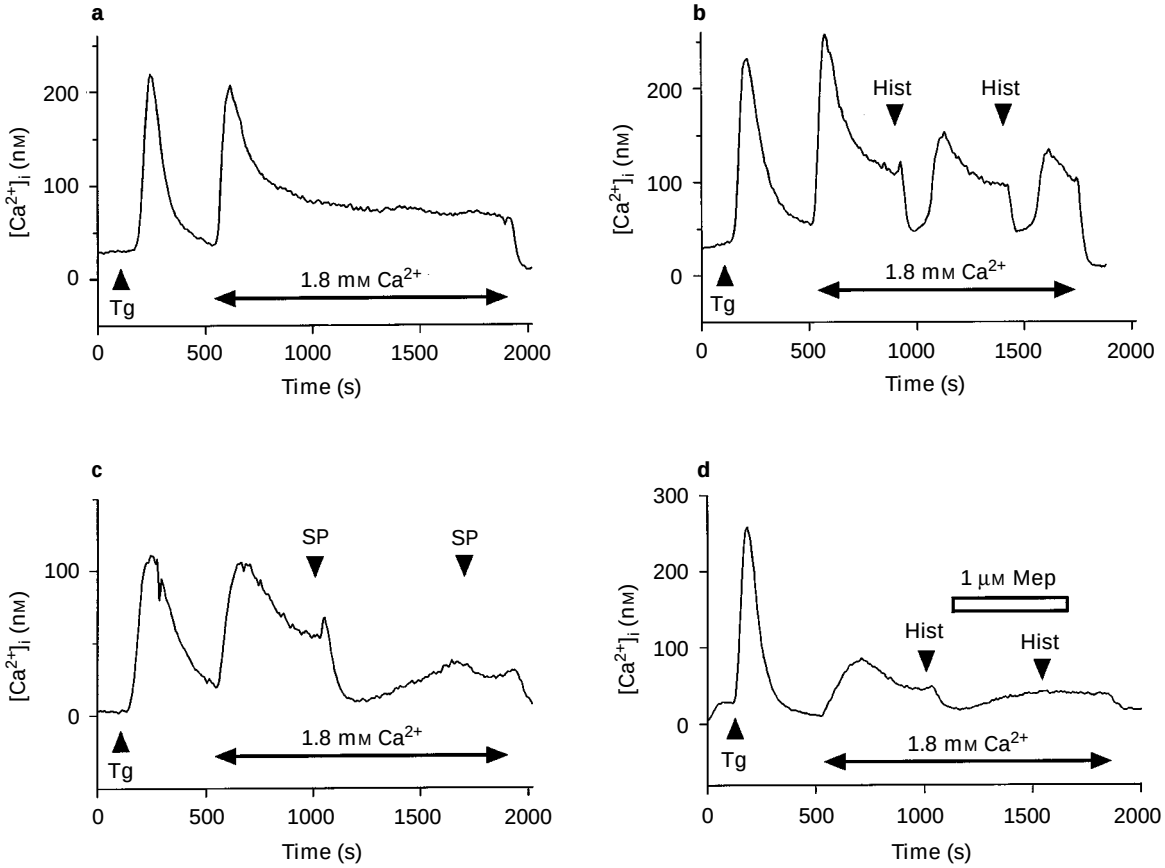

Figure 3 Reduction of steady-state $[Ca^{2+}]_i$ by histamine and substance P in thapsigargin-treated U373 MG cells. Cells, grown to confluency on coverslips and preloaded with fura-2, were treated with 5 μM thapsigargin (Tg) in Ca^{2+}-free HEPES buffer before continuous perfusion with HEPES containing 1.8 mM Ca^{2+}. (a) No agonist added after the readdition of Ca^{2+}. (b) Additions of 100 μM histamine (Hist) were for 100 s. (c) Additions of 10 nM substance P (SP) were for 100 s. (d) Additions of 100 μM histamine were for 100 s. Mepyramine (Mep), 1 μM, was present from 100 s after the first addition of histamine until 100 s after the second histamine addition, as indicated by the horizontal bar. The traces are representative of 3 (b, c and d) or 27 (a) measurements.

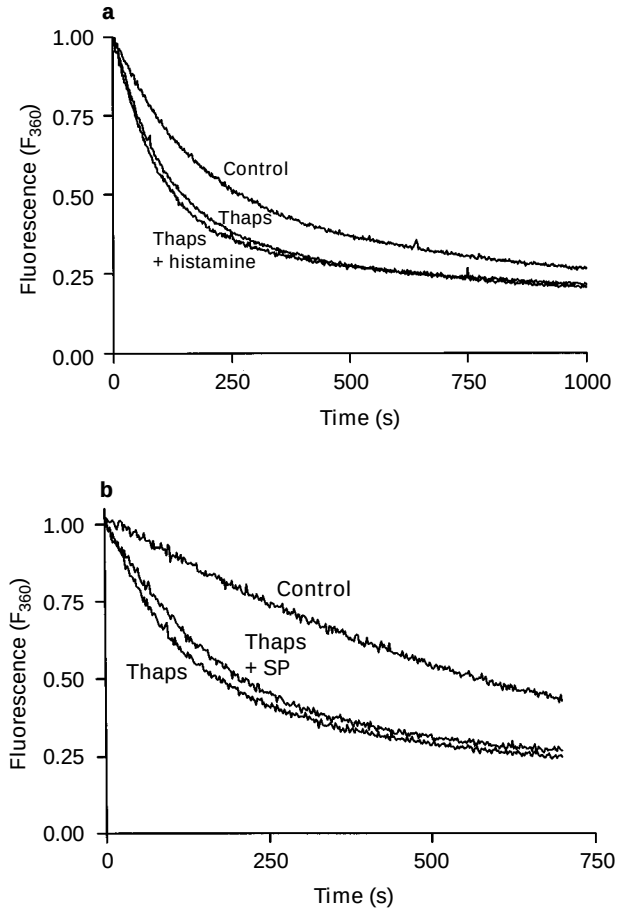

Figure 5 Quench of fura-2 fluorescence by Mn^{2+} in U373 MG cells. Cells, grown to confluency on coverslips and preloaded with fura-2, were continuously perfused with HEPES buffer to which no Ca^{2+} had been added. Where indicated, cells were preincubated with 5 μM thapsigargin (thaps) in 0 Ca^{2+} HEPES for 15 min before the start of the experiment. The ordinate scale is the relative fluorescence intensity measured at 360 nm. (a) The best-fit values of the rate constant for quench of fura-2 fluorescence by 750 μM Mn^{2+} (see Methods for calculation) were $4.25 \pm 0.03 \times 10^{-3}$ s^{-1} for control cells, and $5.36 \pm 0.02 \times 10^{-3}$ s^{-1} and $5.60 \pm 0.02 \times 10^{-3}$ s^{-1} for thapsigargin and thapsigargin + 100 μM histamine, respectively. Traces are representative of 6 or 7 experiments. (b) The rate constants for quench of fura-2 fluorescence by 250 μM Mn^{2+} were $1.44 + 0.12 \times 10^{-3}$ s^{-1} for control cells and $4.71 \pm 0.71 \times 10^{-3}$ s^{-1} and $5.02 \pm 0.35 \times 10^{-3}$ s^{-1} for thapsigargin and thapsigargin + 10 nM substance P (SP), respectively. Traces are representative of 4 experiments.

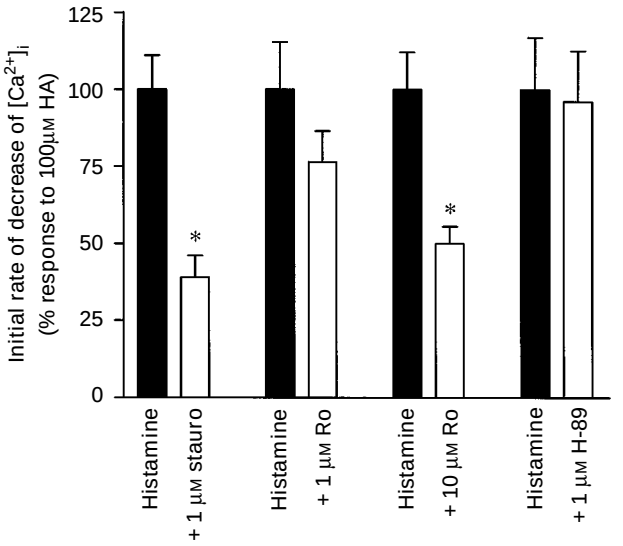

Figure 6 Inhibition of histamine-induced decreases in steady-state $[Ca^{2+}]_i$ by protein kinase inhibitors. U373 MG cells, grown to confluency on coverslips and preloaded with fura-2, were treated with 5 μM thapsigargin in Ca^{2+}-free HEPES buffer before continuous perfusion with HEPES containing 1.8 mM Ca^{2+}. Cells were preincubated with 1 mM staurosporine (stauro) for 900 s before the start of the experiment, or were perfused with Ro 31-8220 (Ro) or H-89 for 1100 s, before the addition of histamine (100 μM for 100 s). Values are the mean ± s.e.mean from 4 experiments with staurosporine, 6 and 3 experiments with 1 and 10 μM Ro 31-8220, respectively, and 4 experiments with H-89, and are expressed as a percentage of the response to 100 μM histamine which was measured concomitantly. * Statistically different from control values (Student's t test, $P < 0.05$).

apparatus, reduced the depressant effect of 100 μM histamine on $[Ca^{2+}]_i$ by 61 ± 7% ($P < 0.05$, $n = 4$) (Figure 6). Perfusion with the PKC antagonist Ro 31-8220 (1 μM) for 1100 s reduced the effect of 100 μM histamine (in the continued presence of 1 μM Ro 31-8220) by 24 ± 10% (not significantly different from control response by Student's t test, $n = 6$) (Figure 6). Perfusion with 10 μM Ro 31-8220 for the same period reduced the depressant effect of 100 μM histamine by 50 ± 6% ($P < 0.05$, $n = 3$) (Figure 6). A 1100 s perfusion with the protein kinase A antagonist H-89 (1 μM) did not alter the depressant effect of 100 μM histamine (4 ± 17% reduction when compared to controls, $n = 4$) (Figure 6).

Effect of histamine and forskolin on cyclic AMP accumulation in U373 MG cells

The lack of effect of H-89 and the demonstration that the effect of histamine on $[Ca^{2+}]_i$ after thapsigargin pretreatment is mediated by the H_1-receptor argues strongly against the involvement of cyclic AMP. This was confirmed by direct measurement. Basal cyclic AMP accumulation in dissociated U373 MG cells over a 10 min incubation period was 14.6 ± 0.8 pmol per 10^6 cells ($n = 3$) and was not significantly increased in the presence of 100 μM histamine, 15.2 ± 0.3 pmol per 10^6 cells ($n = 3$). In contrast, the accumulation in the presence of 10 μM forskolin, a direct stimulant of adenylyl cyclase, was 130 ± 24 pmol per 10^6 cells ($n = 3$).

Effect of protein kinase inhibitors on [^{3}H]-mepyramine binding to membranes of guinea-pig cerebellum and on histamine-induced accumulation of [^{3}H]-IP$_1$ in U373 MG cells

The selectivity of protein kinase C inhibitors is limited and the inhibitory effects of Ro 31-8220 and staurosporine on histamine-induced reduction in $[Ca^{2+}]_i$ could reflect a direct action at the H_1-receptor. To assess the likelihood of such an interaction, measurements were made of the ability of Ro 31-8220 and staurosporine to block [^{3}H]-mepyramine binding to H_1-receptors in guinea-pig cerebellar membranes; a model system without the technical problems associated with the use of U373 MG cell membranes (Arias-Montaño *et al.*, 1994). Neither staurosporine (1 μM) nor the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) inhibitor KN-62 (10 μM) had any significant effect on the H_1-receptor binding of [^{3}H]-mepyramine (1 ± 2 and 2 ± 2% inhibition, respectively, $n = 3$), but with 1 and 10 μM Ro 31-8220 specific [^{3}H]-mepyramine binding was inhibited by 17 ± 1 and 55 ± 2%, respectively ($n = 3$).

In view of the inhibitory action of 10 μM Ro 31-8220 on [^{3}H]-mepyramine binding, measurements were made of the

effect of Ro 31-8220 on histamine-induced [^{3}H]-IP_1 accumulation in U373 MG cells, in order to see whether inhibition was apparent in this system. Under the conditions used [^{3}H]-IP_1 accumulation is linear with time and dependent on continued H_1-receptor stimulation (Arias-Montaño *et al*., 1994), so that the degree of inhibition will be independent of the period of stimulation. These experiments were carried out at 37°C, since the rate of [^{3}H]-IP_1 accumulation induced by histamine is markedly reduced at 22°C (Bootman *et al*., 1996), the temperature at which the fluorescence measurements were made. However, we have shown previously that the affinity of antagonists for the H_1-receptor is relatively insensitive to temperature (Wallace & Young, 1983), although equilibration is appreciably faster at 37°C than at 22°C (Wallace & Young, 1983; Treherne & Young, 1988).

Neither 1 nor 10 μM Ro 31-8220 had any significant effect on [^{3}H]-IP_1 accumulation acting alone, nor did they produce any inhibition of [^{3}H]-IP_1 accumulation stimulated by 100 μM histamine (mean accumulations, expressed as a percentage of the response to histamine alone ± approximate s.e.mean, 142 ± 24 and 161 ± 28% in the presence of 1 and 10 μM Ro 31-8220, respectively). The variability of the effect of Ro 31-8220 between experiments was apparent in the size of the errors, but in 2 experiments out of 3 there was a significant potentiation of the response. If there is an inhibitory action at the H_1-receptor, then it is offset by a potentiation of [^{3}H]-IP_1 accumulation consequent on removal of a feedback inhibition via PKC at the level of the receptor, G protein or PLC-β. Consistent with this, 1 μM staurosporine, which was without effect on [^{3}H]-mepyramine binding, produced a statistically significant potentiation of the response to histamine in 3 of 4 experiments (mean response ± approximate s.e.mean 172 ± 26% of that with histamine alone). In this series of experiments with staurosporine the final concentration of DMSO was 4%, but the same concentration of DMSO was present in 3 experiments with 10 μM KN-62, which produced no significant change in the response to histamine (86 ± 8% of the response to histamine alone). Basal accumulations containing the same concentration of DMSO were subtracted before comparison of responses (see Methods). The Ro 31-8220 used in this series of experiments was dissolved in distilled water. The conclusion to be drawn from these experiments is that, although Ro 31-8220 may have some direct effect at the H_1-receptor, there is no detectable inhibition of histamine-stimulated phosphoinositide hydrolysis.

Effect of protein kinase inhibitors on the time-course of histamine-stimulated increases in $[Ca^{2+}]_i$ in U373 MG cells

The evidence above indicates that micromolar concentrations of Ro 31-8220 do not inhibit histamine-induced phosphoinositide hydrolysis, but the interpretation of experiments such as those illustrated in Figure 6 must still be approached with caution. To gain further information, measurements were also made of the effect of protein kinase inhibitors on the time-course of histamine-induced Ca^{2+} mobilization. If PKC plays a role in accelerating the return of the histamine-induced increase in $[Ca^{2+}]_i$ to resting levels, then inhibition of PKC activation should alter the rate at which the initial peak $[Ca^{2+}]_i$ declines to prestimulated levels. In the absence of extracellular Ca^{2+} the time-course of the Ca^{2+} signal is not complicated by any Ca^{2+} entry component. This should minimize effects of PKC inhibition on the rate of H_1-receptor desensitization, although any re-release of Ca^{2+} taken back up into 1,4,5-IP_3-sensitive stores stimulated by histamine might still be affected.

Pretreating the cells with 1 μM Ro 31-8220 for 20 min in Ca^{2+}-containing HEPES buffer before mounting in the cuvette and perfusing with HEPES buffer to which no Ca^{2+} had been added did not significantly alter the initial peak response to 100 μM histamine in the continued presence of 1 μM Ro 31-8220 (451 ± 107 and 513 ± 118 nM increases in the presence and absence of the inhibitor, $n = 3$) (Figure 7a). In contrast, 1 μM Ro 31-8220 slowed the rapid decrease in $[Ca^{2+}]_i$ which occurred immediately after the initial peak histamine-stimulated Ca^{2+} release, such that the total Ca^{2+} activity in response to 100 μM histamine (measured as the area under the trace) was increased significantly by 70 ± 14% (% increase over a paired control response as described under Methods, $n = 3$) (Figure 7a). Pretreatment with 10 μM Ro 31-8220 also had no significant effect on the peak increase in $[Ca^{2+}]_i$ in response to 100 μM histamine (409 ± 51 nM and 476 ± 47 nM increases in the absence and presence of 10 μM Ro 31-8220, respectively, $n = 3$), but the area under the trace was significantly increased by 80 ± 11% (Figure 7b).

The Ca^{2+}/calmodulin-dependent protein kinase (CaMK) has been implicated in the homologous desensitization of histamine H_1-receptors in the GT1-7 neuronal cell line (Zamani & Bristow, 1996). In HEPES buffer to which no Ca^{2+} had been added, a 20 min pretreatment with 10 μM KN-62, a selective CaMK inhibitor did not alter the initial peak increase in $[Ca^{2+}]_i$ stimulated by 100 μM histamine (4 ± 8% decrease in the presence of KN-62, $n = 4$) (Figure 7c). The total area under the Ca^{2+} trace was numerically increased by KN-62 by 25 ± 8%, but the effect was not statistically significant. However, it is notable that the action of KN-62 on the second, slower phase of reduction in $[Ca^{2+}]_i$ (Figure 7c) was reproducible between experiments. KN-62 (10 μM) did significantly increase resting $[Ca^{2+}]_i$ by 12 ± 2 nM ($n = 4$).

Consistent with the failure to detect any significant formation of cyclic AMP by histamine and with the lack of inhibition of the depressant effect of histamine on $[Ca^{2+}]_i$ in thapsigargin-treated U373 MG cells (Figure 6), a 20 min pretreatment period with the PKA inhibitor H-89 (1 μM) had no significant effect on the extent or time-course of 100 μM histamine-induced increases in $[Ca^{2+}]_i$ in U373 MG cells (Figure 7d).

Effect of histamine on the thapsigargin-induced plateau phase of $[Ca^{2+}]_i$ in HeLa cells

To investigate whether the dual effect of histamine in stimulating both Ca^{2+} mobilization and Ca^{2+} extrusion was a peculiarity of U373 MG cells or whether it could be observed in other cell lines, measurements were also made on human HeLa cells, since H_1-receptor mediated [^{3}H]-IP formation and Ca^{2+} mobilization responses have been extensively characterized in this line (Tilly *et al*., 1990; Bristow *et al*., 1991; Bootman *et al*., 1996). Thapsigargin (5 μM), in the absence of extracellular Ca^{2+}, stimulated a transient 169 ± 10 nM increase in $[Ca^{2+}]_i$ in HeLa cells. Readdition of 1.8 mM Ca^{2+} to the perfusion buffer caused a 216 ± 15 nM increase in $[Ca^{2+}]_i$ which then declined to a plateau level 46 ± 4 nM above prestimulated levels (measured 300 s after the addition of Ca^{2+}, $n = 6$). The application of histamine (100 μM, 100 s) produced a consistent decline in this level, although in experiments in which the plateau was not marked the magnitude of the response was small (Figure 8).

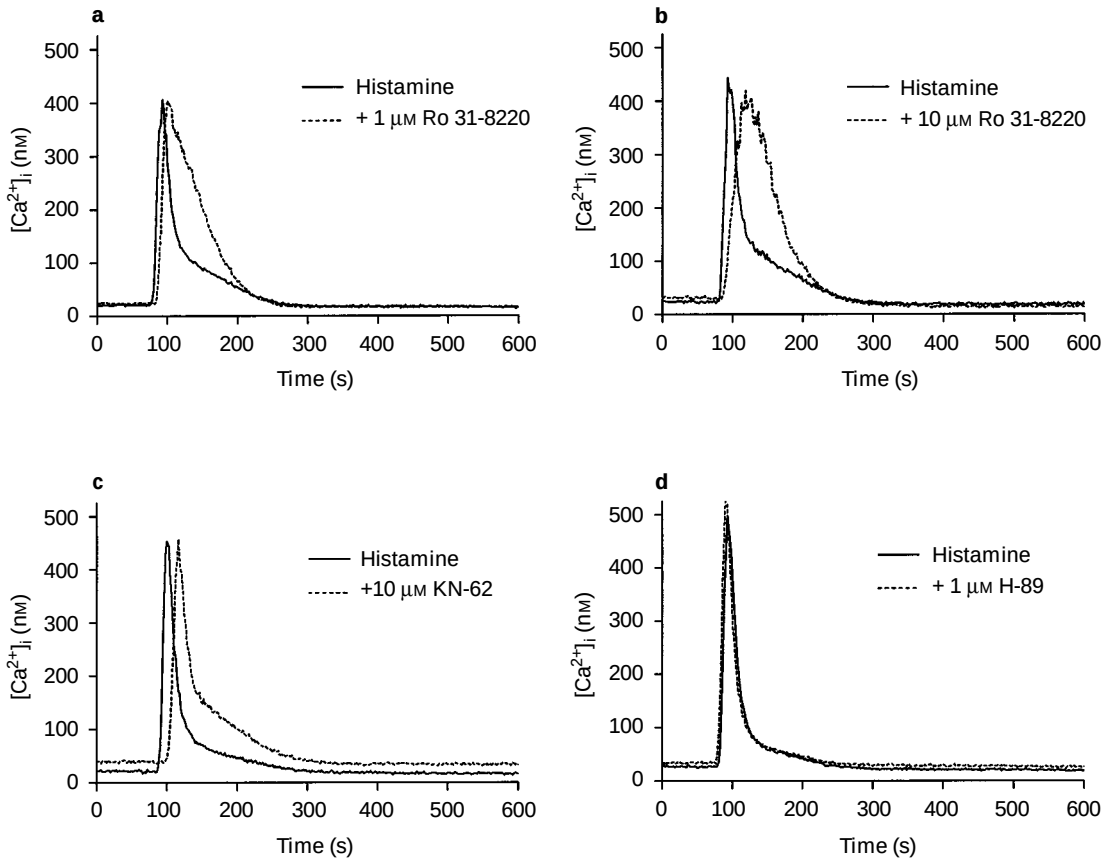

Figure 7 Effect of protein kinase inhibitors on histamine-stimulated increase in $[Ca^{2+}]_i$ in U373 MG cells. Cells, grown to confluency on coverslips and preloaded with fura-2, were perfused continuously with HEPES with no added Ca^{2+}. Coverslips were pretreated with kinase inhibitor for 20 min before mounting in the fluorometer. Histamine (100 μM alone or histamine + kinase inhibitor were included in the perfusion buffer from 60 s onwards. (a) 1 μM Ro 31-8220, (b) 10 μM Ro 31-8220, (c) 10 μM KN-62 and (d) 1 μM H-89. Traces are representative of 3 (a,b,d) or 4 (c) experiments.

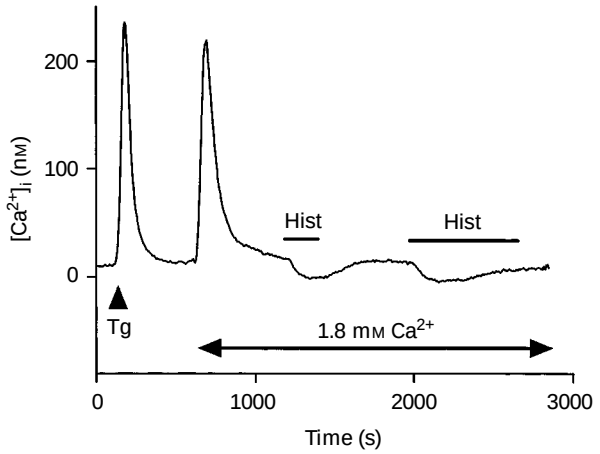

Figure 8 Effect of histamine on $[Ca^{2+}]_i$ in thapsigargin-treated HeLa cells. HeLa cells, grown to confluency and preincubated with fura-2 AM as described under Methods, were treated with 5 μM thapsigargin (Tg) in HEPES buffer with no added Ca^{2+}, before perfusion with normal Ca^{2+}-containing HEPES. Histamine (Hist, 100 μM) was applied for the periods indicated by the horizontal lines. The experiment was repeated twice further with similar results.

Discussion

The main finding of this study is that histamine and substance P not only stimulate Ca^{2+} mobilization via the phosphoinositide signalling pathway, but also activate an homeostatic mechanism which facilitates the return of $[Ca^{2+}]_i$ to prestimulated levels. This is most clearly apparent after pretreatment with thapsigargin, which leads to the emptying of intracellular stores and the stimulation of store-refilling-induced Ca^{2+} entry (Putney, 1990; Berridge, 1995). The 'plateau' level of $[Ca^{2+}]_i$ after thapsigargin represents a steady state between Ca^{2+}-entry and Ca^{2+}-extrusion and the action of histamine and substance P to reduce this level must reflect either an inhibition of Ca^{2+} entry or a stimulation of Ca^{2+} extrusion, or both. There is evidence in the literature that both processes are open to modulation by agonists, but the results of the present study indicate that in U373 MG cells it is Ca^{2+} efflux that is affected.

Ca^{2+} influx in the presence of thapsigargin appears to be largely via store-refilling activated Ca^{2+} channels, consistent with the increased rate of quench of fura-2 fluorescence by Mn^{2+} in the presence of thapsigargin (Figure 5). There is evidence from decreases in the rate of the Mn^{2+} quench that Ca^{2+} entry via this pathway is inhibited by thrombin in human umbilical vein endothelial cultures (Neylon & Irvine, 1991) and by *N*-formyl-methionyl-leucyl-phenylalanine in human neutrophils (Montero *et al*., 1993). This inhibitory effect on Ca^{2+} entry in neutrophils, but not in endothelial cells, was partially inhibited by staurosporine and mimicked by phorbol esters, suggesting an involvement of PKC in neutrophils, but not in endothelial cells, although the regulation of capacitative Ca^{2+} entry by PKC can be complex (Petersen & Berridge, 1994). However, the failure of either histamine or substance P to

decrease the rate of quench of Fura-2 in U373 MG cells makes it probable that the induced decrease in $[Ca^{2+}]_i$ is an effect on Ca^{2+} extrusion rather than on Ca^{2+} entry.

Ca^{2+} extrusion from cells can involve both the plasma membrane Ca^{2+} pump and the Na^+-Ca^{2+} exchanger (Carafoli, 1994; Monteith & Roufogalis, 1995). There is evidence that the latter may play a role in limiting peak Ca^{2+} mobilization by agonists in rat cultured astrocytes (see Takuma *et al*., 1996), but at low $[Ca^{2+}]_i$ it is probable that the high affinity/low capacity Ca^{2+} pump is much the more important mechanism (Carafoli, 1994; Monteith & Roufogalis, 1995). There are data in the literature showing that the activity of the Ca^{2+} pump can be stimulated by PKC activation (Balasubramanyam & Gardner, 1995; reviewed by Monteith & Roufogalis, 1995) and the evidence that the effects of histamine and substance P on $[Ca^{2+}]_i$ in thapsigargin-treated U373 MG cells are mediated by H_1- and NK_1-receptors, respectively, which are coupled to phosphoinositide hydrolysis and DAG production, would be consistent with the PKC pathway. Protein kinase A (PKA) is clearly not involved, since not only was the PKA inhibitor H-89 without effect, but there was no detectable stimulation of cyclic AMP accumulation by 100 μM histamine. However, the interpretation of the effects of protein kinase C inhibitors is more complex.

The inhibition of histamine-induced decreases of $[Ca^{2+}]_i$ in thapsigargin-treated cells by staurosporine (Figure 6) is consistent with the involvement of PKC, but staurosporine is notably non-selective as an inhibitor of protein kinases and has other actions, notably an apparent inhibition of Ca^{2+} efflux from human neutrophils (Wong *et al*., 1992). Ro 31-8220 is a much more selective PKC inhibitor, but may have an action at the H_1-receptor. At 1 μM, a concentration 100 fold greater than the IC_{50} for PKC quoted by the supplier, there was no significant inhibition by Ro 31-8220 of the decrease in $[Ca^{2+}]_i$ induced by histamine in thapsigargin-treated cells (Figure 6). However, the K_1 value of 3 nM against PKC was determined from the inhibition of the partially purified enzyme from rat brain (Davis *et al*., 1992), whereas in intact cell systems the IC_{50} values seem to be nearer 1 μM, e.g. as measured against antigen-driven T-cell proliferation (Davis *et al*., 1992) or carbachol-induced acid secretion in parietal cells (McKenna & Hanson, 1993). It must also be borne in mind that the potency of PKC inhibitors can vary between forms of PKC, as observed with indolocarbazoles (Martiny-Baron *et al*., 1993) and staurosporine (McGlynn *et al*., 1992), and that it is not known which PKC isozymes are present in U373 MG cells. However, with 10 μM Ro 31-8220, which produces a significant inhibition of the histamine-induced fall in $[Ca^{2+}]_i$ there is also an appreciable inhibition of the binding of [^{3}H]-mepyramine to H_1-receptors on guinea-pig cerebellar membranes. Measurement of binding to H_1-receptors on membranes prepared from U373 MG cells requires large amounts of cell material and presents some technical difficulties (Arias-Montaño *et al*., 1994). We have therefore used guinea-pig cerebellar membranes as a more approachable model system, although it has the disadvantage that there can be species differences in the binding of ligands to H_1-receptors (Chang *et al*., 1979; Hill & Young, 1980). The affinity constant for mepyramine is a factor of approximately 5 lower in human HeLa cells (Arias-Montaño & Young, 1993) and human U373 MG cells (Arias-Montaño *et al*., 1994) than in guinea-pig brain (Hill & Young, 1980), so that the extent of the inhibition by Ro 31-8220 at the H_1-receptor might be less in U373 MG cells than on cerebellar membranes. However, even if there is some inhibition at the level of the receptor by 10 μM Ro 31-8220, it might not be significant if there is a receptor reserve for the action of histamine in stimulating extrusion. This is commonly the case with second messenger-mediated effects (Strickland & Loeb, 1981). However, the most powerful argument against any inhibition by 10 μM Ro 31-8220 at the H_1-receptor having any effect on the functional response is that there was no inhibition of histamine-induced [^{3}H]-IP_1 accumulation. Indeed, in two out of three experiments Ro 31-8220 produced a statistically significant potentiation of the response to histamine. This suggests that there is a negative feedback on histamine-stimulated [^{3}H]-IP_1 accumulation via PKC, which is removed in the presence of the PKC inhibitor. Consistent with this interpretation, staurosporine also significantly potentiated histamine-induced [^{3}H]-IP_1 accumulation in 3 out 4 experiments.

The possible involvement of PKC in histamine H_1-receptor desensitization has been investigated in a number of cell lines (reviewed in Hishinuma *et al*., 1996; see also Zamani *et al*., 1995). Interestingly downregulation of PKC by extended exposure to a phorbol ester had no effect on the initial peak Ca^{2+} mobilization by histamine in HeLa cells, but caused an elevation of the subsequent 'plateau' phase (Smit *et al*., 1992). However, whereas acute application of phorbol esters invariably inhibits H_1-receptor mediated-responses, there are few examples where the same responses to histamine are potentiated in the presence of selective PKC inhibitors. Exceptions are in bovine adrenal chromaffin cells, where Ro 31-8220 at a concentration of 10 μM, but not at 1 μM, caused a significant increase (34%) in histamine-induced 1,4,5-IP_3 formation measured at 10 s, although total [^{3}H]-IP accumulation stimulated by histamine over a 30 min period was not altered (Boarder & Challiss, 1992), and in human umbilical vein endothelial cells, in which Ro 31-8220 (10 μM) produced a small (15%), but significant, inhibition of histamine-induced desensitization of H_1-receptor-mediated inositol phosphate formation (McCreath *et al*., 1994). The lack of effect of 1 μM Ro 31-8220 on 1,4,5-IP_3 formation in the adrenal chromaffin cells (Boarder & Challiss, 1992) is similar to the lack of significant inhibition by 1 μM Ro 31-8220 of histamine-stimulated Ca^{2+} efflux (Figure 6), whereas 10 μM Ro 31-8220 was effective in both cases. The lack of inhibition of histamine-stimulated [^{3}H]-IP accumulation by 10 μM Ro 31-8220 is another parallel between the two studies. This is consistent with the observation that 10 μM Ro 31-8220 had no effect on the peak histamine-induced increase in $[Ca^{2+}]_i$ (Figure 7). A similar lack of effect of 10 μM Ro 31-8220 on the peak Ca^{2+} signal induced by 100 μM histamine has been observed in the DDT_1 MF-2 smooth muscle cell line (Dickenson & Hill, 1993). The unaltered peak Ca^{2+} signals are also consistent with a receptor reserve for 1,4,5-IP_3-mediated Ca^{2+} release, as has been previously noted for carbachol- and bradykinin-induced Ca^{2+} mobilization in human SH-SY5Y cells (Willars & Nahorski, 1995). As noted above, this is expected to be the rule for second messenger-mediated responses and it seems likely that a similar reserve exists for the histamine-induced decrease of $[Ca^{2+}]_i$ in thapsigargin-treated cells, although the pathway of the stimulated efflux is more likely to be via diacylglycerol than via 1,4,5-IP_3.

A particular advantage of the experimental protocol using thapsigargin (see Figure 3) is that histamine-induced inhibition of Ca^{2+} extrusion by a protein kinase inhibitor cannot be due to any inhibition of H_1-receptor desensitization, which would serve to increase the effect of histamine. We have attempted to minimize any effect on receptor desensitization on the time-course of histamine-induced Ca^{2+} mobilization (Figure 7) by

omitting Ca^{2+} from the extracellular medium. Refilling of 1,4,5-IP_3-sensitive stores and re-release of Ca^{2+} on continued H_1-receptor activation should thus be limited and confined to Ca^{2+} taken back up into stores before it can be extruded from the cell. An effect of Ro 31-8220 or staurosporine in inhibiting desensitization could still contribute to the reduction in the rate at which $[Ca^{2+}]_i$ returns to resting levels after histamine-stimulated Ca^{2+} release, but it seems likely that the major effect of Ro 31-8220 is in inhibiting histamine-stimulated Ca^{2+} efflux. KN-62, a selective inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Tokumitsu *et al*., 1990), which has been implicated in H_1-receptor desensitization in GT1-7 neuronal cells (Zamani & Bristow, 1996), had no statistically significant effect on the area under the histamine-stimulated ($[Ca^{2+}]_i$) versus time curve. There is an indication of an effect on the secondary, slower declining phase, most marked in the trace reproduced in Figure 7(c), but it is not clear that this is related to receptor desensitization and could represent another action of the drug, particularly since the increase in resting $[Ca^{2+}]_i$ induced by KN-62 cannot be ascribed to an effect on the H_1-receptor. The failure of KN-62 to stimulate histamine-induced [^{3}H]-IP_1 accumulation also argues against an action on H_1-receptor desensitization.

The balance of the evidence with Ro 31-8220 favours the involvement of PKC in the stimulation of Ca^{2+} extrusion by histamine in U373 MG cells. Not only is the effect of histamine in stimulating the decrease of $[Ca^{2+}]_i$ in thapsigargin-treated cells blocked by 10 μM Ro 31-8220, but even at 1 μM there is a significant effect on the secondary phase of histamine-induced Ca^{2+} mobilization. However, it is clear that the time-course of changes in $[Ca^{2+}]_i$ is subject to several interlinking controls, each of which is individually subject to modulatory influences, which may differ between cultures. This appears to apply with some force to U373 MG cells, since the pattern of histamine-induced Ca^{2+} mobilization in Ca^{2+}-containing medium was not invariable and in some cultures there was a 'plateau' (Figure 2b), similar to that observed in suspensions of U373 MG cells (Hishinuma *et al*., 1996). However, the ability of histamine or substance P to decrease the thapsigargin-induced plateau level of increased $[Ca^{2+}]_i$ in confluent monolayers of cells also disappeared at certain times and there appeared to be some correlation with periods when the histamine-stimulated Ca^{2+}-mobilization also did not return to prestimulated levels (Figure 2b). This is consistent with the lack of effect of histamine on $[Ca^{2+}]_i$ after thapsigargin in U373 MG cells, evident in traces recorded during a study of histamine-induced γ-aminobutyric acid (GABA) release (Soria-Jasso & Arias-Montaño, 1996), where the plateau phase of histamine-induced Ca^{2+} mobilization was even more marked than that in Figure 2(b). Biphasic Ca^{2+} responses, such as that shown in Figure 2(a) have also been recorded in response to ATP in intact rat aortic endothelium and have been shown to be critically dependent on the membrane potential (Usachev *et al*., 1995). This underlines the complexity of the control of $[Ca^{2+}]_i$ observed. Small variations in the activity of ion channels or enzymes could well have a marked effect on the time-course and pattern of changes in $[Ca^{2+}]_i$. In the present study growing the cells for 24 h in DMEM/F-12 medium to which no foetal bovine serum had been added often led to the return of the inhibitory responses to histamine and substance P on $[Ca^{2+}]_i$. Therefore, it seems possible that batch variations in the serum may be one factor in altered responses to agonists, perhaps by a desensitization of protein kinase-mediated effects (see Freshney, 1994).

In summary, we have demonstrated that in human U373 MG astrocytoma cells, agonists linked to phosphoinositide hydrolysis have a dual effect on $[Ca^{2+}]_i$: an initial stimulation as a consequence of release from intracellular stores and a secondary activation of a homeostatic mechanism whereby $[Ca^{2+}]_i$ is returned towards prestimulated levels. This reduction in $[Ca^{2+}]_i$ most likely occurs via activation of a Ca^{2+} extrusion pump and the balance of the experimental evidence suggests that it is mediated by PKC. Whilst this extrusion process may be regarded as a safety mechanism preventing excessive increases in $[Ca^{2+}]_i$, it may also be important to maintain the temporal aspects of the Ca^{2+} signal within the U373 MG cell. Indeed, the original two pool model for cytoplasmic Ca^{2+} oscillations (Berridge, 1991) required that Ca^{2+} be extruded from the cell at the end of a Ca^{2+} spike event. It is also clear that inhibition of Ca^{2+} entry, and hence inhibition of store refilling, as observed with *N*-formyl-methionyl-leucyl-phenylalanine in human neutrophils (Montero *et al*., 1993), will have markedly different effects on regenerative Ca^{2+} spikes than a process which reduces $[Ca^{2+}]_i$ by stimulating extrusion.

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