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Characteristics of the Ca^{2+}-dependent inhibition of cyclic AMP accumulation by histamine and thapsigargin in human U373 MG astrocytoma cells

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1 Histamine, acting on H_1-receptors, caused a Ca^{2+}-dependent inhibition of forskolin- and isoprenaline-induced cyclic AMP accumulation in monolayers of human U373 MG cells (IC_{50} 1.3 ± 0.3 μM, maximum inhibition $66 \pm 3\%$). The inhibition was not reversed by the protein kinase inhibitor K-252A.

2 Thapsigargin also inhibited cyclic AMP accumulation (IC_{50} 6.0 ± 0.3 nM, maximum inhibition $72 \pm 1\%$). In the absence of extracellular Ca^{2+} 5 μM thapsigargin caused only a $12 \pm 2\%$ inhibition of cyclic AMP accumulation.

3 The inhibitory effect of 100 nM thapsigargin on forskolin-stimulated cyclic AMP accumulation was blocked by La^{3+} (best-fit maximum inhibition $81 \pm 4\%$, IC_{50} 125 ± 8 nM). In contrast, the inhibitory action of 10 μM histamine was much less sensitive to reversal by 1 μM La^{3+} ($33 \pm 5\%$ reversal, compared with $78 \pm 6\%$ reversal of the inhibition by thapsigargin measured concurrently). However, in the presence of both thapsigargin and histamine the inhibition of cyclic AMP accumulation was reversed by 1 μM La^{3+} to the same extent as the inhibition by thapsigargin alone.

4 Thapsigargin (5 μM) + 1 μM La^{3+} caused only a $20 \pm 1\%$ inhibition of histamine-stimulated phosphoinositide hydrolysis.

5 There was no indication from measurement of intracellular Ca^{2+} of any persistent La^{3+}-insensitive Ca^{2+} entry component activated by histamine.

6 The results provide evidence that Ca^{2+} entry is required for the inhibition by histamine and thapsigargin of drug-induced cyclic AMP accumulation in U373 MG astrocytoma cells. The differential sensitivity of the inhibitory action of the two agents to block by La^{3+} suggests that more than one pathway of Ca^{2+} entry is involved.

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; [^{3}H]-IP, total [^{3}H]-inositol phosphates; IBMX, 3-isobutyl-1-methylxanthine; IL-1β, interleukin-1β; IL-6, interleukin-6; PKC, protein kinase C

Introduction

The human U373 MG astrocytoma cell line has been widely used as a model system for the study of the activation of astrocytes by cytokines, particularly by interleukin-1β (IL-1β) and tumour necrosis factor α (Ballestas & Benveniste, 1997; Lieb *et al*., 1996; Carlson & Aschmies, 1995 and references therein). The primary signalling pathway for cytokines is *via* a kinase cascade (Carlson & Aschmies, 1995; Eder, 1997), but responses may be modulated by both the cyclic AMP and Ca^{2+}/protein kinase C (PKC) signalling pathways. Thus in U373 MG cells agents increasing cyclic AMP inhibit IL-1β-induced cell proliferation (Kasahara *et al*., 1990) and the expression of mRNA for the adhesion molecules ICAM-1 and VCAM-1 (Ballestas & Benveniste, 1997), but promote IL-1β-stimulated expression of interleukin-6 (IL-6) mRNA (Kasahara *et al*., 1990). IL-1β-stimulated IL-6 release may involve a PKC (Lieb *et al*., 1996) and agents which stimulate phosphoinositide hydrolysis in U373 MG cells, and hence Ca^{2+} mobilization and activation of PKC, can themselves stimulate IL-6 release (Cadman *et al*., 1994) and immediate early gene expression (Eistetter *et al*., 1992).

We have observed that thapsigargin and histamine, in the presence of a phosphodiesterase inhibitor, produce a Ca^{2+}-dependent inhibition of forskolin-stimulated cyclic AMP accumulation in U373 MG cells. There are several mechanisms by which 'cross-talk' between the Ca^{2+}/PKC and cyclic AMP pathways can occur (reviewed by Selbie & Hill, 1998), but it is only relatively recently that the action of Ca^{2+} on the Ca^{2+}-inhibitable isoforms of adenylyl cyclase has been explored in detail (reviewed by Cooper *et al*., 1995; Mons & Cooper, 1995; Antoni, 1997; Housley & Milligan, 1997). A special feature of this interaction, as determined in C6-2B glioma cells and HEK 293 cells transfected with type VI adenylyl cyclase, is that it is primarily Ca^{2+} entry through store-refilling activated channels which is effective in producing inhibition of cyclase activity (Cooper *et al*., 1994; Chiono *et al*., 1995; Fagan *et al*., 1998). This implies a close spatial apposition of Ca^{2+} entry channels and Ca^{2+}-inhibitable cyclase and means that measurement of bulk cytoplasmic $[Ca^{2+}]_i$ may not reflect the concentration in the immediate vicinity of the cyclase, as has been demonstrated by expression of a type VI adenylyl cyclase-aequorin construct

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in HEK 293 cells (Nakahashi *et al*., 1997). The inhibition of agonist-stimulated cyclic AMP accumulation by drugs stimulating Ca^{2+} entry is therefore not only of interest as a mechanism for the regulation of U373 MG cell function, but also because it allows a more direct determination of which Ca^{2+} entry pathways are involved in the modulation of a specific cellular response. We report here a study of the characteristics of the inhibition of forskolin- and isoprenaline-induced cyclic AMP accumulation in U373 MG cells by thapsigargin and by histamine. Some of the results have been presented in preliminary form to the British Pharmacological Society (Wong *et al*., 1998; Wong & Young, 1999a,b).

Methods

Measurement of [^{3}H]-cyclic AMP accumulation

Intracellular cyclic AMP was measured using the [^{3}H]-adenine labelling method of Salomon *et al*. (1974). U373 MG astrocytoma cells were cultured and dissociated as described previously (Young *et al*., 1998a). The cells were seeded onto 6-well culture plates at a density of approximately 7×10^4 cells well^{-1} and grown to near confluence. The cells were incubated in DMEM F-12 medium (3 h, 37°C) containing [2-^{3}H]-adenine (3 μCi well^{-1}) to label the intracellular ATP pool. The labelling medium was aspirated and the cells were equilibrated in HEPES-buffered medium with no added Ca^{2+} (in mM): NaCl, 152.5; KCl, 5.4; NaH_2PO_4, 1; $MgSO_4$, 0.8; L-glutamine, 0.6; D-glucose, 5; HEPES, 5; pH 7.4) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min at 37°C. K-252a, mepyramine, pirdonium, propranolol and La^{3+} were added at this stage. In experiments in which La^{3+} was present NaH_2PO_4 was omitted from the medium. Incubation with forskolin or isoprenaline (normally 1 or 4 min), with or without thapsigargin or histamine, was in HEPES-buffered medium containing 1.8 mM Ca^{2+} or in medium with no added Ca^{2+}. In experiments in which extracellular Ca^{2+} was reduced to a very low level by addition of 0.2 mM EGTA, parallel incubations contained 2 mM Ca^{2+} + 0.2 mM EGTA, to give a free Ca^{2+} concentration of 1.8 mM. Incubations were terminated by addition of 5% trichloroacetic acid (final concentration) to each sample. The plates were left on ice for 30 min and the reaction mixtures then centrifuged at $13{,}000 \times g$ for 3 min to sediment cell debris. [γ-^{32}P]-ATP (10,000–20,000 c.p.m.), unlabelled cyclic AMP (100 μl, 10 mM) and unlabelled ATP (10 μl, 65 mM) were added to the supernatants, which were then loaded onto Dowex AG50W-X4 (200–400 mesh; Sigma) columns and [^{3}H]-ATP eluted with 4 ml distilled water. A further 6.5 ml distilled water was added and the eluant loaded directly onto a neutral alumina column (Sigma), which was washed with 4 ml 0.1 M imidazole buffer, pH 8. The columns were eluted with a further 4 ml imidazole buffer to give a fraction containing [^{3}H]-cyclic AMP. Scintillator (Opti-fluor; Packard, 16 ml) was added and ^{3}H/^{32}P determined by scintillation counting. Corrections were made for recovery of the ATP (as indicated by the recovery of [^{32}P]-ATP) and cyclic AMP (determined from the O.D. of the eluate from the alumina column). The results were expressed as [^{3}H]-cyclic AMP recovered $\times$ 100/[^{3}H]-ATP.

Measurement of intracellular Ca^{2+} concentration

Measurements of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in U373 MG cells grown on coverslips and loaded with fura-2 were made as described previously (Young *et al*., 1998a) using a Hitachi F-2000 fluorimeter. The coverslips were superfused with a modified HEPES medium at 37°C with or without added Ca^{2+} and with or without La^{3+}. The perfusion was stopped for the period that thapsigargin was present.

Histamine-induced [^{3}H]-inositol phosphate accumulation

U373 MG cells were seeded (approximately 50,000 cells well^{-1}) onto 12-well plates (Costar) and grown to near confluence. The culture medium was removed and the monolayers washed with 1 ml inositol-free DMEM before addition of 0.5 ml inositol-free DMEM containing 10% dialyzed calf serum, 10 μM *myo*-inositol and 2.5 μCi ml^{-1} [^{3}H]-inositol (0.16 μM). After 20–24 h, the labelling medium was aspirated and the cells were equilibrated for 10 min at 37°C in 1.0 ml HEPES medium. HEPES medium containing LiCl (30 mM) was added (0.48 ml) to each well and the cells incubated for 15 min at 37°C before addition of agonist in a 20 μl volume. Cells were then incubated for a further 4 min and reactions terminated by aspirating the medium, rinsing each well with 1 ml ice-cold HEPES buffer containing LiCl, and adding 0.5 ml 10% perchloric acid, containing 1 mM EDTA and 1 mg ml^{-1} phytic acid. The plates were left to stand on ice for 30 min. A 450 μl aliquot was taken from each well and added to 400 μl of a 1 : 1 (v : v) solution of trioctylamine/1,1,2-trichlorotrifluorethane. [^{3}H]-Inositol phosphates were separated as described previously (Young *et al*., 1998a).

Analysis of data

Concentration-response data for isoprenaline-induced cyclic AMP accumulation and the blockade by La^{3+} of the inhibition by thapsigargin of forskolin-induced cyclic AMP accumulation 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 $\text{Resp}_{\max}$ is the maximum response, C is the concentration isoprenaline, 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. Data for the inhibition of forskolin-induced cyclic AMP accumulation by thapsigargin and histamine were fitted to the equation

$$\%\ \text{of control} = (100 - \text{Inh}_{\max}) / ((A/IC_{50})^{n_H} + 1) + \text{Inh}_{\max}$$

where $\text{Inh}_{\max}$ is (100 − the maximum percentage inhibition), A is the concentration of histamine or thapsigargin, nH is the Hill coefficient and IC_{50} is the concentration of histamine or thapsigargin giving 50% inhibition of the histamine- or thapsigargin-sensitive cyclic AMP accumulation.

Statistical comparison of parameters characterizing two concentration response curves was made by fitting the curves simultaneously and assessing the increase in the residual sum of squares when parameters were constrained to be the same for both curves, as described previously (Young *et al*., 1998a). Where multiple comparisons of means were made within the same experiment the significance of differences between conditions was made using the Student–Newman–Keuls multiple range test, following one-way analysis of variance. The errors of ratios were calculated using the approximate formula, i.e. the coefficient of variation of the ratio is equal to the square root of the sum of the squares of the coefficients of variation of the numerator and denominator (Colquhoun, 1971). The overall mean of a series of

measurements, each of which provided a mean ± s.e.mean, was expressed as the weighted mean ± s.e.mean (Colquhoun, 1971).

Chemicals

[8-^{3}H]-Adenine (26 Ci mmol^{-1}) was obtained from Amersham International and [γ-^{32}P]-ATP (6000 Ci mmol^{-1}) from New England Nuclear. *Myo*-[2-^{3}H]-inositol, 18–24 Ci mmol^{-1}, (New England Nuclear) was diluted 1 : 10 with distilled H_2O and passed down a Dowex AG 1-X8 column before use. ATP, cyclic AMP, dimethylsulphoxide (DMSO), EDTA, fura-2 acetoxymethyl ester, L-glutamine, histamine dihydrochloride, HEPES, 3-isobutyl-1-methylxanthine (IBMX), isoprenaline sulphate, lanthanum chloride, mepyramine maleate, perchloric acid, phytic acid, (±)-propranolol, thapsigargin, 1,1,2-trichlorotrifluoroethane (freon), tri-n-octylamine and Tris were purchased from Sigma. Forskolin was obtained from Calbiochem, K-252a from Alexis Corp. and trichloracetic acid from Fisons. Pirdonium bromide was kindly provided by Prof H. Timmerman, Vrije Universiteit, Amsterdam.

Stock solutions of fura-2, IBMX and K-252a were prepared in DMSO. The maximum concentration of DMSO present in any assay was 1% (v : v). Forskolin was dissolved in 95% ethanol.

Results

Ca^{2+}-dependent inhibition of cyclic AMP accumulation in U373 MG cells by histamine

Isoprenaline induced a concentration-dependent accumulation of cyclic AMP in U373 MG cells in the presence of the non-selective phosphodiesterase inhibitor IBMX (0.5 mM), with a best-fit EC_{50} of 0.13 ± 0.01 μM (Hill coefficient 1.02 ± 0.05) (Figure 1A), measured after a 1 min incubation. The accumulation induced by 1 μM isoprenaline reached a maximum after approximately 4 min and remained at this level over the next 6 min (Figure 1B), the longest period for which measurements were made. The response to 1 μM isoprenaline was blocked by the non-selective β-adrenoceptor antagonist propranolol (0.1 μM) (94 ± 1 and 97 ± 1% inhibition with 1 and 4 min incubation with isoprenaline, respectively; two independent experiments at each time).

Histamine (10 μM) acting alone had no effect on cyclic AMP accumulation, consistent with our earlier observations on dissociated U373 MG cells (Young *et al*., 1998a), but produced a marked inhibition of the response to isoprenaline (Figure 2). The inhibitory effect of histamine was completely dependent on the presence of Ca^{2+} in the extracellular medium. Reduction of extracellular Ca^{2+} to a very low level by the omission of Ca^{2+} from the medium and the addition of 0.2 mM EGTA had no effect on the response to isoprenaline alone, but abolished the inhibition produced by histamine in Ca^{2+}-containing medium (Figure 2). The effect of histamine on the concentration-response curve to isoprenaline was to decrease the maximum response (Figure 1A), without any significant effect on the EC_{50} or the Hill coefficient.

The inhibition of the response to 1 μM isoprenaline by 10 μM histamine in Ca^{2+}-containing medium was reversed by 2 μM pirdonium (to 95 ± 2% of the response to isoprenaline alone, $n = 2$) and by 1 μM mepyramine (to 94 ± 4%, $n = 2$), indicating that the response to histamine is mediated by H_1-receptors.

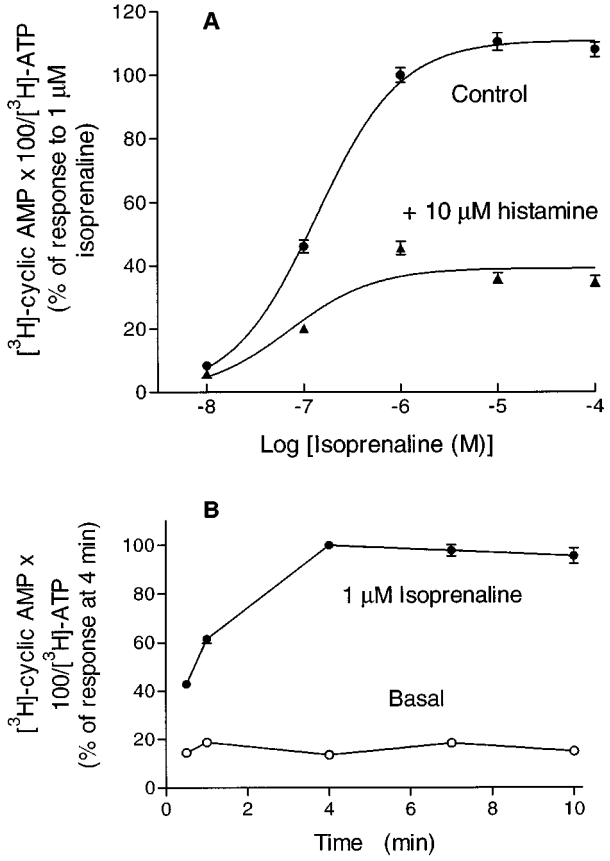

Figure 1 Isoprenaline-induced cyclic AMP accumulation in U373 MG cells. (A) Concentration-dependence and effect of 10 μM histamine. Points are the weighted means ± s.e.mean from six independent experiments (3–6 determinations at each concentration). Incubations were for 1 min. To allow for variations in the magnitude of the stimulation of cyclic AMP accumulation between experiments, response has been expressed as a percentage of the response to 1 μM isoprenaline acting alone (mean stimulation 4.1 ± 0.1 fold of basal), which was measured in every experiment. (B) Time-course of cyclic AMP accumulation induced by 1 μM isoprenaline. Responses are expressed as the percentage of the response to isoprenaline at 4 min, which was measured in every experiment (mean stimulation 7.3 ± 0.2 fold of basal, $n = 6$). Other points are the weighted means ± s.e.mean from three determinations.

The Ca^{2+}-dependent inhibition of cyclic AMP accumulation appears to be at the level of the cyclase, since histamine also inhibited the accumulation induced by forskolin, a directly-acting stimulator of the cyclase (Figure 3A). The time-course of cyclic AMP accumulation induced by 10 μM forskolin was similar to that for 1 μM isoprenaline (Figure 1A), except that accumulation induced by forskolin slowed rather than ceased completely after *circa* 4 min (Figure 3A). However, the extent of the inhibition by 10 μM histamine of the responses to forskolin and isoprenaline was established rapidly (significant difference between inhibition measured over 30 s and 1 min only against isoprenaline) and was closely similar at all times between 30 s and 10 min (Figure 3B).

The inhibition by histamine of cyclic AMP accumulation induced by 1 μM isoprenaline was concentration-dependent (Figure 4), with a best-fit IC_{50} of 1.3 ± 0.3 μM (Hill coefficient 0.96 ± 0.26) and a best-fit maximum inhibition in this series of experiments of 66 ± 3%.

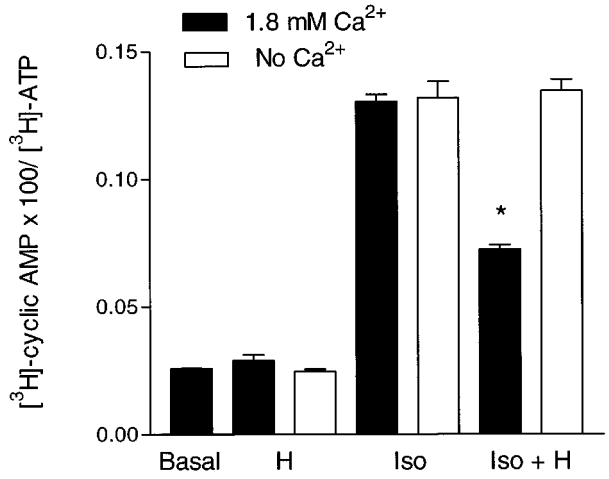

Figure 2 Ca^{2+}-dependence of the inhibition of isoprenaline-stimulated cyclic AMP accumulation by histamine. EGTA (0.2 mM) was present in every incubation, either with or without the addition of 2 mM Ca^{2+}. Incubation with 1 μM isoprenaline (Iso) $\pm$ 10 μM histamine (H) was for 4 min. The values are means $\pm$ s.e.mean from five replicate determinations within a single experiment. The whole experiment was repeated twice further. $^*P<0.001$ compared to isoprenaline with Ca^{2+}.

Effect of protein kinase inhibition on the action of histamine on cyclic AMP accumulation

Extracellular Ca^{2+} is essential for the inhibition of cyclic AMP accumulation by histamine (Figure 2), but G protein-mediated activation of PLC is markedly inhibited by the removal of extracellular Ca^{2+} (see e.g. Wojcikiewicz *et al*., 1994; Arias-Montaño *et al*., 1994), leaving the possibility that the inhibitory action of histamine might be mediated by protein kinase C (PKC). However, the broad-spectrum protein kinase inhibitor K-252a, 1 μM, did not prevent the inhibition of the response to isoprenaline by 10 μM histamine (Figure 5). The very small effect of K-252A could be accounted for by the increase in the response to isoprenaline in the presence of K-252a (120 $\pm$ 4% of the response to 1 μM isoprenaline alone, $n=4$; statistically significant increase in three experiments) (Figure 5). K-252a, 1 μM, had no significant effect on the basal accumulation of cyclic AMP.

Ca^{2+}-dependent inhibition of isoprenaline-induced cyclic AMP accumulation by thapsigargin

Thapsigargin, 5 μM, which causes the release of Ca^{2+} from intracellular stores cells and the activation of store-refilling-linked Ca^{2+} entry in U373 MG cells (Young *et al*., 1998a), also inhibited cyclic AMP accumulation induced by 1 μM isoprenaline in Ca^{2+}-containing medium, measured over a 1 min incubation period (Figure 6). However, in a medium in which free extracellular Ca^{2+} was reduced to negligible levels by the omission of Ca^{2+} and the addition of 0.2 mM EGTA, the inhibition by thapsigargin was only 12 $\pm$ 2% (Figure 6) (statistically significant inhibition in two out of four experiments), although the increase in $[Ca^{2+}]_i$ due to emptying of intracellular stores by 5 μM thapsigargin is maximal at this time (Young *et al*., 1998a). The response to isoprenaline alone was not significantly altered in the EGTA medium (97 $\pm$ 3% of control). After a 2 min incubation with 1 μM isoprenaline (response in the EGTA medium 100 $\pm$ 3% of control) the inhibition by 5 μM thapsigargin in the EGTA medium was 11 $\pm$ 2% ($n=3$, inhibition statistically significant in only one experiment). The inhibition by thapsigargin in the presence of extracellular Ca^{2+} in these experiments was 49 $\pm$ 2% ($n=4$, 1 min incubation) and 66 $\pm$ 2% ($n=3$, 2 min incubation). Thapsigargin alone had no significant effect on basal cyclic AMP accumulation at either time point.

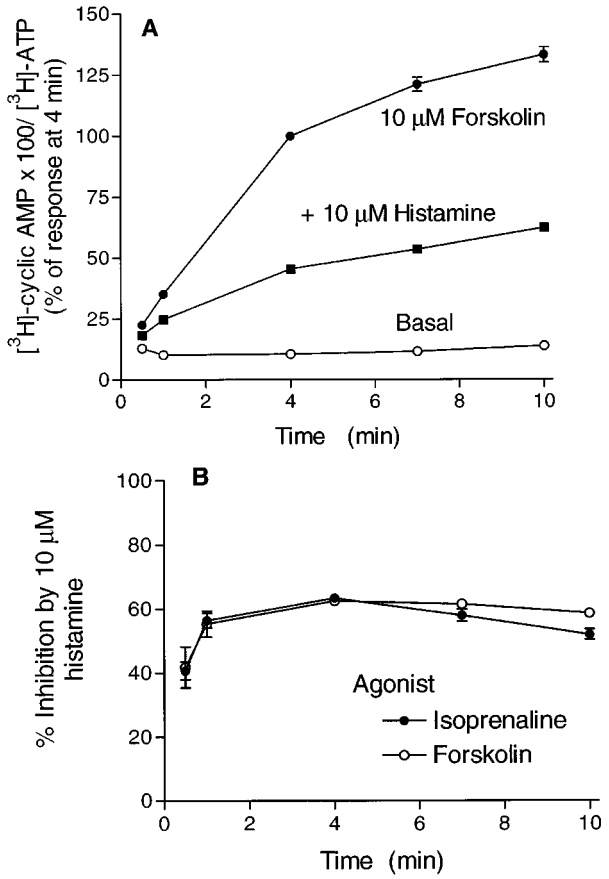

Figure 3 Effect of histamine on forskolin- and isoprenaline-stimulated cyclic AMP accumulation as a function of time. (A) Time course of cyclic AMP accumulation stimulated by 10 μM forskolin in the presence and absence of 10 μM histamine. To allow for variations in the response between experiments, the response to forskolin after 4 min incubation has been set equal to 100 (mean stimulation 8.9 $\pm$ 0.2 fold of basal, $n=6$). (B) Variation with time of the inhibition by 10 μM histamine of the responses to 1 μM isoprenaline and 10 μM forskolin. In both panels the points represent the means $\pm$ approximate s.e.mean of three determinations (five with isoprenaline at 10 min). Basal cyclic AMP accumulation has been subtracted. Where no error bars are apparent the error was within the size of the symbol.

The Ca^{2+}-dependent inhibition of cyclic AMP accumulation by thapsigargin, like that by histamine, was independent of the agent used to stimulate the cyclase, since the response to 10 μM forskolin was also inhibited. The concentration-dependence of the inhibition by thapsigargin is shown in Figure 7. The best-fit value of the IC_{50} was 6.0 $\pm$ 0.3 nM and the best-fix maximum inhibition 72 $\pm$ 1%. The best-fit value of the Hill coefficient was 2.44 $\pm$ 0.16, probably reflecting the effectively irreversible nature of the action of thapsigargin (Treiman *et al*., 1998).

Effect of La^{3+} on the inhibition of cyclic AMP accumulation by thapsigargin and histamine

Store-refilling activated Ca^{2+} channels in rat mast cells (Hoth & Penner, 1993), Jurkat cells (Aussel *et al*., 1996) and SH-SY5Y neuroblastoma cells (Grudt *et al*., 1996) are reported to

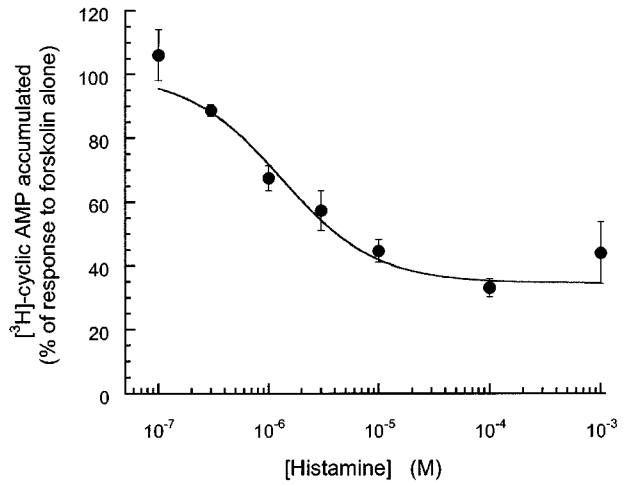

Figure 4 Concentration-dependence of the inhibition by histamine of isoprenaline-stimulated cyclic AMP accumulation. Points are the weighted means ± approximate s.e.mean from 2–7 determinations of the ratio of cyclic AMP accumulation in the presence of antagonist to the accumulation in the presence of 1 μM isoprenaline alone (1 min incubation). The curve drawn is the best-fit line to a Hill equation (see Methods).

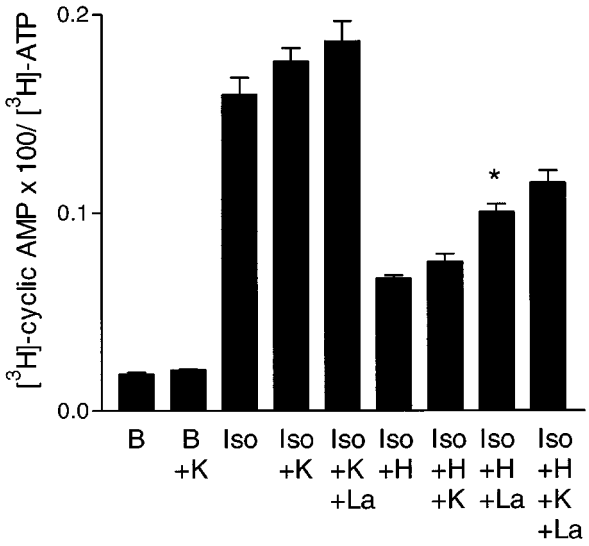

Figure 5 Effect of protein kinase blockade by K-252A on the inhibition of isoprenaline-stimulated cyclic AMP accumulation by histamine. The bars represent the mean ± s.e.mean of quadruplicate determinations within a single experiment in the presence 1 μM isoprenaline (Iso) with or without 1 μM K-252A (K), 10 μM histamine (H) and 1 μM La^{3+} (La), or in the absence of isoprenaline (Basal, B). The whole experiment was repeated twice further with this combination of treatments. *Significantly different from isoprenaline + histamine, $P<0.001$. There was no significant difference between the pairs: basal and basal + K-252A, isoprenaline and isoprenaline + K-252A, isoprenaline + K-252A and isoprenaline + K-252A + La^{3+}, isoprenaline + histamine and isoprenaline + histamine + K-252A, isoprenaline + histamine + La^{3+} and isoprenaline + histamine + La^{3+} + K-252A.

be particularly sensitive to blockade by low concentrations of La^{3+}. The channels in U373 MG cells also appear to be very sensitive to La^{3+}, since the increased level of intracellular Ca^{2+} induced by treatment with 100 nM thapsigargin in Ca^{2+}-free medium followed by re-addition of Ca^{2+} was rapidly reduced to low levels by 1 μM La^{3+} (Figure 8A). Consistent with an involvement of Ca^{2+} entry through these channels in the inhibition of cyclic AMP accumulation by thapsigargin, the

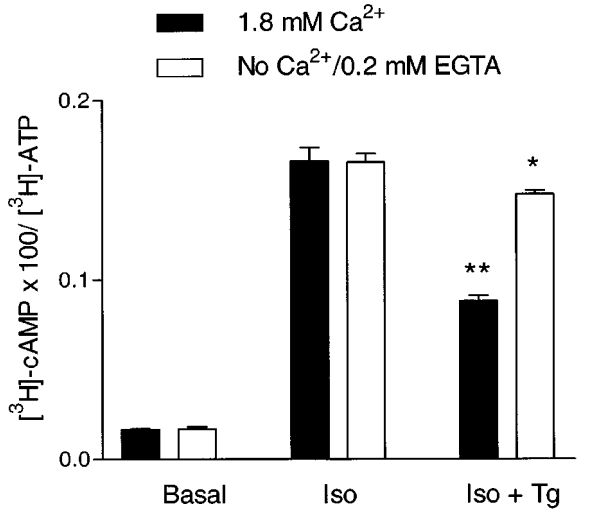

Figure 6 Inhibition of isoprenaline-stimulated cyclic AMP accumulation by thapsigargin: contribution of Ca^{2+} released from intracellular stores. Incubation with 1 μM isoprenaline (Iso) + 5 μM thapsigargin (Tg) in normal Ca^{2+}-containing medium or in medium with no added Ca^{2+} and containing 0.2 mM EGTA was for 1 min. The data are the means ± s.e.mean of six replicate determinations within a single experiment, which was repeated a further three times. *Significantly different from isoprenaline in the absence of Ca^{2+}, $P<0.01$. **Significantly different from isoprenaline + Ca^{2+}, $P<0.001$.

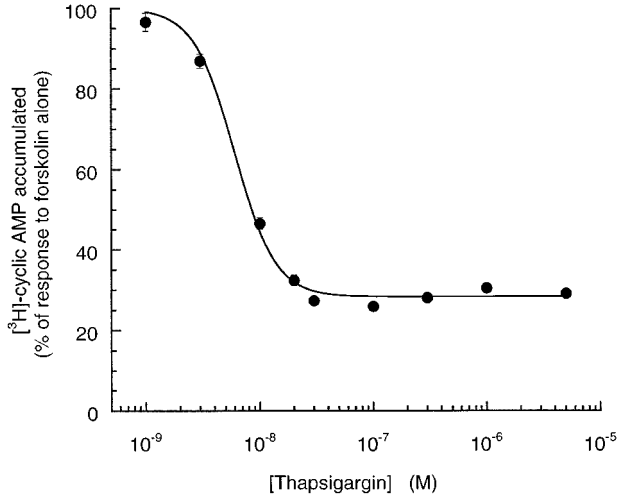

Figure 7 Concentration-dependence of the inhibition by thapsigargin of forskolin-stimulated cyclic AMP accumulation in U373 MG cells. Incubation with 10 μM forskolin + thapsigargin was for 4 min. Points are the weighted means ± s.e.mean of 2–6 independent determinations. The curve drawn is the best-fit line to a Hill equation (see Methods).

effect of 100 nM thapsigargin on cyclic AMP accumulation stimulated by 10 μM forskolin was inhibited by La^{3+} in a concentration-dependent manner (Figure 9), although the reversal of the effect of thapsigargin was not complete (maximum of the best-fit curve 85.2 ± 1.1%). With the foot of the curve set at 28.9% (the mean inhibition in the presence of thapsigargin alone), this represents a maximum reversal of the effect of thapsigargin of 81 ± 1%. This may be slightly overestimated, since 1 μM La^{3+} consistently produced a small stimulation of the response to 10 μM forskolin, although the increase was statistically significant in only two out of seven determinations (mean stimulation 110 ± 2%). The best-fit IC_{50} for La^{3+} was 125 ± 8 nM.

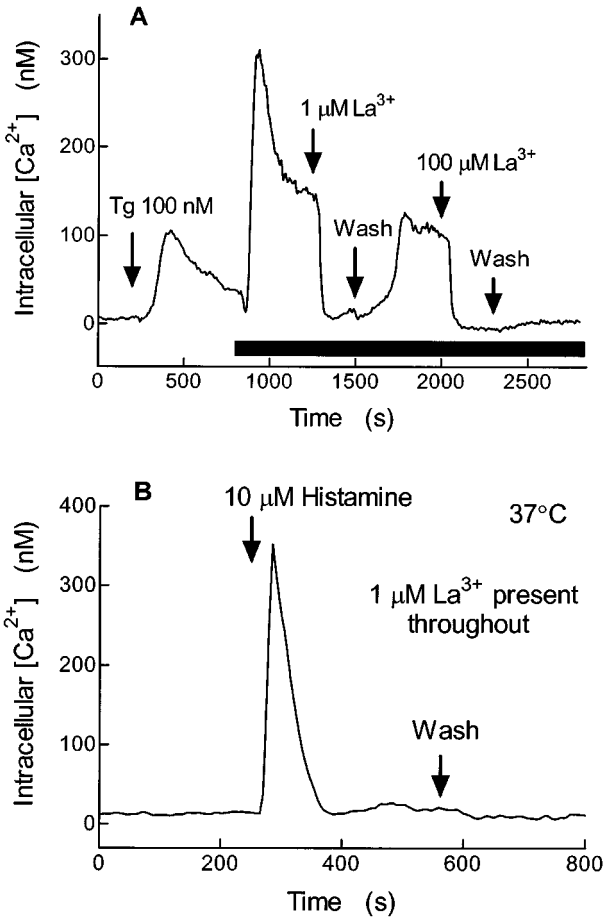

Figure 8 Effect of La^{3+} on the increases in $[Ca^{2+}]_i$ in a monolayer of U373 MG cells induced by thapsigargin and histamine. (A) Thapsigargin (100 nM) was added in medium without added Ca^{2+}. The cells were superfused with medium containing 1.8 mM Ca^{2+} for the period indicated by the filled horizontal bar. (B) Histamine (10 μM). Ca^{2+} (1.8 mM) and 1 μM La^{3+} were present throughout. The traces in A and B are from single experiments, which were repeated twice further.

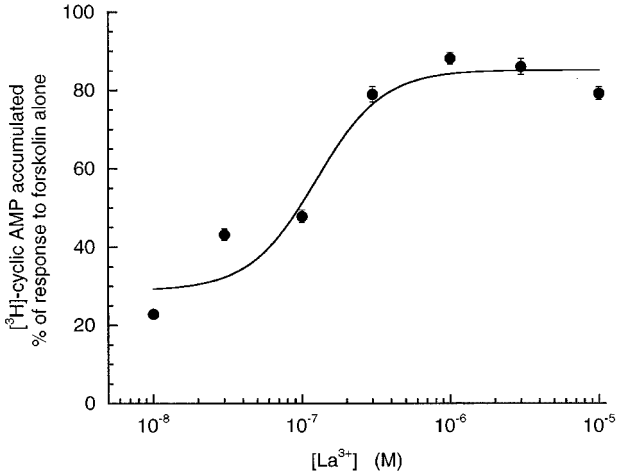

Figure 9 Concentration-dependence of the reversal by La^{3+} of the inhibition by 100 nM thapsigargin of cyclic AMP accumulation stimulated by 10 μM forskolin. The cells were incubated with La^{3+} for 10 min before addition of forskolin and further incubation for 4 min. Points are the weighted means from 3–6 independent determinations. The mean stimulation by forskolin was 9.3±0.2 fold of basal (n=9). The curve drawn is the best-fit line to a Hill equation (see Methods), with the foot of the curve set to 28.9%, the weighted mean inhibition in the absence of La^{3+}.

In contrast to the effect on the inhibition by 100 nM thapsigargin, the inhibitory effect of 10 μM histamine on cyclic AMP accumulation induced by 10 μM forskolin was only weakly blocked by 1 μM La^{3+} (35±3% reversal, n=4). Similarly, 1 μM La^{3+} only partly reversed the inhibition by 10 μM histamine of isoprenaline-induced cyclic AMP accumulation (29±3% reversal, n=7). The effect of La^{3+} on the extent of the inhibition by histamine was not significantly altered in the presence of the protein kinase inhibitor K-252A (Figure 5).

The differential effect of 1 μM La^{3+} on the inhibitory actions of thapsigargin and histamine was further apparent when direct comparison was made within the same experiment (Table 1). The extent of the effect of 1 μM La^{3+} was more variable in this series of experiments, but the difference between the mean extent of the reversal of the inhibition by 100 nM thapsigargin and that by 10 μM histamine was statistically significant and clear. This suggests that the pathways of Ca^{2+} entry activated by thapsigargin and histamine are different. Surprisingly, however, in each of the three experiments 1 μM La^{3+} was significantly more effective at reversing the inhibitory effect of histamine when 100 nM thapsigargin was also present (mean in Table 1). In each experiment there was no significant difference between the extent of the inhibitions by histamine or thapsigargin acting alone and that produced by 10 μM histamine and 100 nM thapsigargin in combination. The same was true in three experiments in which the concentration of thapsigargin was increased to 5 μM.

The greater effect of La^{3+} in reversing the inhibition of forskolin-stimulated cyclic AMP accumulation by thapsigargin compared with that due to histamine appeared to be more pronounced when the concentration of thapsigargin was increased to 5 μM (Table 1), although there was no significant difference in the inhibitory effect of 1 μM La^{3+} on the inhibitions by 10 μM histamine in combination with 100 nM or 5 μM thapsigargin in three experiments in which direct

Table 1 Comparison of the effect of 1 μM La^{3+} in reversing the inhibition of forskolin-stimulated cyclic AMP accumulation by histamine and by thapsigargin

	% reversal by 1 μM La^{3+} of inhibition of cyclic AMP accumulation by thapisigargin and/or histamine	
Inhibitor	*A*	*B*
Thapisigargin (100 μM)	78±6	–
Thapisigargin (5 μM)	–	102±2
Histamine (10 μM)	33±5	35±3
Histamine + 100 μM thapisigargin	61±8	–
Histamine + 5 μM thapisigargin	86±9	100±2

Values are the weighted means±s.e.mean from two independent series of experiments (three experiments in A and four in B), in which direct comparisons were made. La^{3+} was added 10 min before 10 μM forskolin + inhibitor. Incubations with forskolin were for 4 min. Cylic AMP accumulation induced by 10 μM forskolin alone was set to 100%. The inhibition produced by histamine + 5 μM thapsigargin was not determined in these experiments, but was taken to be the same as that produced by histamine alone, mean 59±2% in series B, since there was no difference between the two in a separate series of experiments (both 43±2% inhibition of forskolin-stimulated cyclic AMP accumulation, n=3). The inhibition due to 5 μM thapisigargin in B was 66±2%. The mean inhibitions produced by 100 μM thapsigargin, 10 μM histamine and histamine + 100 μM thapisigargin in series A were 57±2, 50±2 and 54±2, respectively.

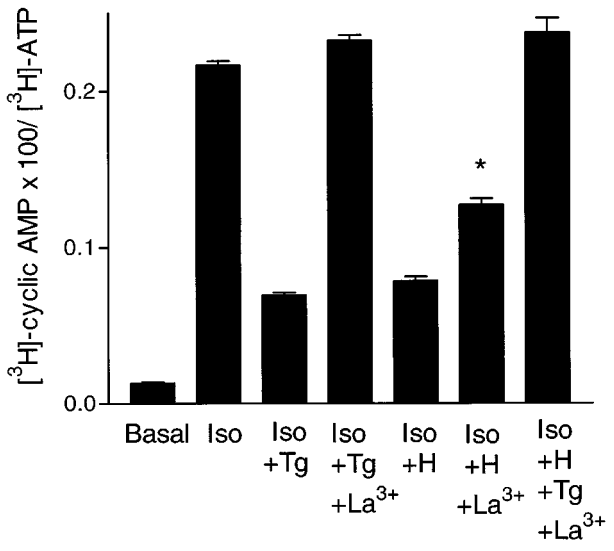

Figure 10 Differential effect of La^{3+} on the inhibition by histamine and thapsigargin of cyclic AMP accumulation induced by a 4 min incubation with isoprenaline. Isoprenaline (1 μM) (Iso), 5 μM thapsigargin (Tg), 10 μM histamine (H) and 1 μM La^{3+} were present where indicated. Points are the means ± s.e.mean from five replicate determinations within a single experiment. Ca^{2+} (1.8 mM) was present throughout. The whole experiment was repeated once further. *Significantly different from isoprenaline + histamine, $P<0.01$. There was no significant difference between the pairs: isoprenaline and isoprenaline + thapsigargin + La^{3+}, isoprenaline + thapsigargin and isoprenaline + histamine, isoprenaline + thapsigargin + La^{3+} and isoprenaline + thapsigargin + histamine + La^{3+}.

comparison was made (Table 1, under A). There was similarly no significant difference when tested (Student's *t*) over all measurements made ($n=6$ with 100 nM and $n=11$ with 5 μM thapsigargin).

The differential effect of 1 μM La^{3+} on the inhibitions by histamine and thapsigargin was also apparent when cyclic AMP accumulation was stimulated by 1 μM isoprenaline. In the presence of 5 μM thapsigargin and 1 μM La^{3+} the response was 108 ± 3% of that to isoprenaline alone ($n=4$), compared with 29 ± 3% reversal of the response to histamine alone ($n=7$). The results from one of two experiments in which direct comparison was made of the effect of La^{3+} on the inhibitions by histamine and thapsigargin is shown in Figure 10. The greater effect of 1 μM La^{3+} in reversing the inhibition by histamine + thapsigargin compared with that of histamine alone is also apparent. La^{3+} (1 μM) had no significant effect on the response to 1 μM isoprenaline alone (cyclic AMP accumulation 99 ± 4% of that in the absence of La^{3+}, $n=3$).

Effect of thapsigargin and La^{3+} on histamine-stimulated phosphoinositide hydrolysis

The greater blockade by La^{3+} of the inhibitory action of histamine + thapsigargin compared to the blockade of the inhibition produced by histamine alone, could be explained simply if thapsigargin acted in some way to inhibit H_1-receptor activation. We have tested for this by measuring the effect of 5 μM thapsigargin and 1 μM La^{3+}, separately and in combination, on total [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation stimulated by 10 μM histamine over a 4 min incubation in U373 MG cells prelabelled with [^{3}H]-inositol.

Thapsigargin alone produced a small stimulation of [^{3}H]-IP accumulation, 114 ± 1% of the accumulation with no additions ($n=3$), whereas 1 μM La^{3+} had a small inhibitory action on basal accumulation (14 ± 1% inhibition, $n=3$). The effect of 5 μM thapsigargin and 5 μM thapsigargin + 1 μM La^{3+} on [^{3}H]-IP accumulation stimulated by 10 μM histamine over a 4 min incubation was in both cases to cause only a small, but significant, inhibition (14 ± 1% and 20 ± 1% inhibition, respectively).

The effect of La^{3+} on changes in $[Ca^{2+}]_i$ induced by histamine

The pattern of changes in $[Ca^{2+}]_i$ induced by histamine in monolayers of U373 MG cells varies between cultures, but usually consists of a rapid initial peak (EC_{50}) for histamine 5 ± 2 μM), which falls to near resting levels after the initial peak, but may be followed by one or more transient increases in $[Ca^{2+}]_i$ (Young *et al*., 1998a,b). This makes it difficult to measure effects on the 'plateau phase' observed in other cell types. However, in the presence of 1 μM La^{3+} 10 μM histamine produced only a transient increase in $[Ca^{2+}]_i$ (Figure 8B), mean peak 299 ± 30 nM ($n=3$), which presumably reflects release from intracellular stores, since it is still observed in the presence of 1 mM EGTA (Young *et al*., 1998a). There was no evidence of any persistent Ca^{2+} influx, which might indicate histamine-stimulated Ca^{2+} entry through channels insensitive to 1 μM La^{3+}.

Discussion

The marked Ca^{2+}-dependent inhibition by histamine and thapsigargin of isoprenaline-stimulated cyclic AMP accumulation in the presence of 0.5 mM IBMX, a non-selective phosphodiesterase inhibitor, suggests that human U373 MG astrocytoma cells possess one or more of the isoforms of adenylyl cyclase which are sensitive to inhibition by induced Ca^{2+} entry. In the human 1321N1 astrocytoma cell line histamine inhibits cyclic AMP accumulation *via* activation of a Ca^{2+}-dependent isoform of phosphodiesterase (Nakahata *et al*., 1986). However, this inhibition is completely reversed by 0.1 mM IBMX (Nakahata *et al*., 1986) and it therefore seems unlikely that the Ca^{2+}-dependent inhibition in U373 MG cells in the presence of 0.5 mM IBMX reflects a change in phosphodiesterase activity. The decrease in the rate of cyclic AMP accumulation after *circa* 4 min may reflect feedback regulation of the cyclase by protein kinase A, as has been reported for both the type V (Iwami *et al*., 1995) and type VI (Chen *et al*., 1997) Ca^{2+}-inhibitable isoforms. Indeed the time-course of the change in type VI cyclase activity in transfected Hi-5 cell membranes (Chen *et al*., 1997) is very similar to the time-course of cyclic AMP accumulation observed here.

The marked inhibition of G protein-mediated activation of PLC when extracellular Ca^{2+} is reduced to a low level (see e.g. Wojcikiewicz *et al*., 1994; Arias-Montaño *et al*., 1994) complicates the interpretation of the Ca^{2+}-dependence of the inhibitory action of histamine. However, there is no evidence that H_1-receptors can couple to G_i (Leopoldt *et al*., 1997) and no indication of PKC-mediated inhibition of cyclic AMP accumulation, such as has been reported for the type VI cyclase (Lai *et al*., 1997), since the broad-spectrum PKC inhibitor K-252A had no effect on the inhibitory effect of histamine. Further, the inhibitory action of thapsigargin provides strong evidence for a Ca^{2+}-dependent inhibition of cyclic AMP accumulation in U373 MG cells. The IC_{50} for thapsigargin for inhibition of forskolin-stimulated cyclic AMP accumulation of 6.0 ± 0.3 nM is lower than the value of approximately 30 nM reported for inhibition of ATP-dependent Ca^{2+} accumulation in permeabilized DDT$_1$MF-2

smooth muscle cells (Bian *et al*., 1991), but the effectively irreversible action of thapsigargin (Treiman *et al*., 1998) makes comparison of measurements made under different experimental conditions difficult.

The marked reduction of the inhibitory action of thapsigargin in the absence of added Ca^{2+} and in the presence of EGTA indicates that Ca^{2+} released from intracellular stores plays only a minor role in inhibition of cyclic AMP accumulation, at most. This is consistent with the conclusion drawn from experiments on HEK 293 cells transfected with the type VI (Cooper *et al*., 1994) and type VIII adenylyl cyclase (Shuttleworth & Thompson, 1999), on native C6-2B glioma cells (Chiono *et al*., 1995; Fagan *et al*., 1998), on bovine adrenal glomerulosa cells (Burnay *et al*., 1998) and on mouse parotid acini (Watson *et al*., 1998) that it is Ca^{2+} entry *via* capacitative entry channels which produces significant modulation of cyclic AMP accumulation.

An observation of particular interest in the present study is that a low concentration of La^{3+}, 1 μM, clearly distinguishes between the action of thapsigargin and that of histamine. The inhibitory action of thapsigargin is largely blocked, whereas the inhibition by histamine is only partly reversed. This difference is observed whether the agent stimulating cyclic AMP accumulation is coupled to adenylyl cyclase *via* Gs (isoprenaline) or activates the cyclase directly (forskolin). The simplest conclusion would seem to be that the pathways of Ca^{2+} entry activated by histamine and thapsigargin differ, although histamine should also activate the capacitative Ca^{2+} entry channels activated by thapsigargin, since there is evidence that the transient increase in $[Ca^{2+}]_i$ produced by histamine in the absence of extracellular Ca^{2+} in U373 MG cells has properties consistent with histamine-induced store release (Young *et al*., 1998b).

There have been a number of studies on other preparations which have demonstrated that agonists at G protein-coupled receptors can stimulate Ca^{2+} entry through two or more channels with differing sensitivity to lanthanides and divalent cations (Clementi *et al*., 1992; Montero *et al*., 1994; van der Zee *et al*., 1995; Kass *et al*., 1994; Kiang, 1997; Iwamuro *et al*., 1998; Broad & Taylor, 1999). There is also good evidence from the cloning of human analogues of the *trp* and *trpl* channels, which are involved in photoreception in *Drosophila* (Hardie & Minke, 1993; Niemeyer *et al*., 1996), for Ca^{2+} permeable channels, which are not activated by thapsigargin, but which can be activated by receptors coupled to $G_{q/11}$ (Okada *et al*., 1998; Boulay *et al*., 1997), including the histamine H_1-receptor (Hofmann *et al*., 1999). It is also notable that these channels, which are most probably non-selective cation channels, are only partially blocked by concentrations of La^{3+} much higher than 1 μM ($\geqslant$50 μM) (Okada *et al*., 1998; Boulay *et al*., 1997). Which of these channels are expressed in U373 MG cells and, if so, whether the coupling between $G_{q/11}$ and Ca^{2+} entry is *via* a direct action of diacylglycerol (Hofmann *et al*., 1999), has not yet been established. There is also evidence that histamine can stimulate Ca^{2+} entry in DDT_1 MF-2 cells *via* the intermediate formation of arachidonic acid (van der Zee *et al*., 1995), which can be derived from diacylglycerol, as in A7r5 cells (Broad & Taylor, 1999), but this seems a less likely pathway for mediating the inhibitory action of histamine in view of the evidence that in HEK 293 cells arachidonic acid-induced Ca^{2+} entry is ineffective in potentiating the activation of the type VIII cyclase, whereas capacitative Ca^{2+} entry does so (Shuttleworth & Thompson, 1999).

It is notable that histamine-induced changes in $[Ca^{2+}]_i$ in populations of U373 MG cells give no indication of a persistent Ca^{2+}-entry component insensitive to 1 μM La^{3+} (Figure 8B). This could reflect a close apposition of Ca^{2+} entry channels and the Ca^{2+} inhibited cyclase, both possibly bound to an anchoring protein analogous to AKAP79 (Sim & Scott, 1999) or INAD (Tsunoda & Zuker, 1999), so that changes in bulk cytoplasmic Ca^{2+} – or lack of – may not reflect the concentration of Ca^{2+} in the immediate vicinity of the cyclase, as has been demonstrated with a type VI cyclase-aequorin construct (Nakahashi *et al*., 1997). The converse is certainly true, namely that increases in $[Ca^{2+}]_i$ do not necessarily lead to inhibition/activation of Ca^{2+}-sensitive cyclases (Chiono *et al*., 1995; Fagan *et al*., 1998; Shuttleworth & Thompson, 1999). In the great majority of studies in which multiple pathways have been identified, such as those cited above (Clementi *et al*., 1992; Montero *et al*., 1994; van der Zee *et al*., 1995; Kass *et al*., 1994; Kiang, 1997; Iwamuro *et al*., 1998; Broad & Taylor, 1999) or those described in Wayman *et al*. (1996) or in Parekh & Penner (1997), measurements were made either of whole cell currents or of changes in $[Ca^{2+}]_i$. It is the great advantage of the approach adopted here that the characteristics of Ca^{2+} entry coupled to inhibition are inferred from the response of the effector. The disadvantage is that indirect measurement potentially allows greater complexity. The particular observation which suggests complexity in the action of thapsigargin or histamine or both is that the ability of La^{3+} to prevent the inhibitory action of histamine is apparently much enhanced when thapsigargin is also present.

The apparent implication of this observation is that the effect of histamine is completely dependent on the release of Ca^{2+} from thapsigargin-sensitive stores, but in this case it would be expected that the effect of histamine should be blocked by 1 μM La^{3+} to the same extent as that of thapsigargin. A direct inhibitory effect of thapsigargin on the H_1-receptor would also explain the observation, but 5 μM thapsigargin only weakly inhibits histamine-induced phosphoinositide hydrolysis. One possible explanation would be that thapsigargin inhibits the histamine-activated Ca^{2+} entry pathway, leaving only the La^{3+}-sensitive pathway activated by emptying of the stores. Such inhibitory effects of thapsigargin have been reported for other channels (Mason *et al*., 1991; Geiszt *et al*., 1995). There is a tendency, although not statistically significant, for the effect of La^{3+} on the combination of histamine + thapsigargin to be more pronounced with 5 μM compared to 100 nM thapsigargin, even though both are on the foot of the inhibition curve for thapsigargin acting alone (Figure 7). This might be an indication of a secondary action of thapsigargin. If there is channel inhibition or promotion of inactivation, then there could in principle be more than one channel activated by the emptying of intracellular stores. We have presented some very preliminary evidence that this may be so (Wong & Young, 1999a). Histamine produces only a small increase in the level of 1,4,5-IP_3 in U373 MG cells (Young *et al*., 1998b), although it is a strong stimulant of phosphoinositide hydrolysis (Arias-Montaño *et al*., 1994). However, the evidence indicates that histamine does mobilize intracellular stores (Young *et al*., 1998b). If thapsigargin, but not histamine, caused the rapid inactivation of a La^{3+}-insensitive capacitative Ca^{2+} entry channel, then La^{3+} would have a differential effect on the two agents, even though each initially activated the same channels. Clearly, the availability of selective inhibitors of the various TRP channels would greatly aid the identification of the pathways involved.

In summary, we show here that there is cross-talk between the Ca^{2+}-mobilizing and cyclic AMP pathways in human U373 MG astrocytoma cells, which may play a role in the

regulation of astrocyte function. The inhibitory effects of histamine and thapsigargin on cyclic AMP accumulation are most probably mediated *via* an action on one or more Ca^{2+} inhibitable isoforms of adenylyl cyclase and appear to involve at least two Ca^{2+} entry pathways with differential sensitivity to La^{3+}.

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