British Journal of Pharmacology (1995) 116, 2715-2723

Characteristics of the binding of [^{3}H]-mepyramine to intact human U373 MG astrocytoma cells: evidence for histamine-induced H_1-receptor internalisation

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1 The kinetics of the binding of 5 nM [^{3}H]-mepyramine to sites on intact human U373 MG astrocytoma cells, sensitive to inhibition by 2 μM pirdonium, were temperature-dependent. At 37°C the half-time for association was 0.9 ± 0.4 min and at 4°C 19 ± 3 min. Dissociation of bound [^{3}H]-mepyramine was fast at 37°C, $t_{0.5}$ 1.5 ± 0.3 min, but at 6°C dissociation initiated by dilution or addition of unlabelled mepyramine was negligible over 120 min. The very slow dissociation at 6°C made it possible to reduce the level of pirdonium-insensitive binding from $56 \pm 5\%$ to $39 \pm 5\%$ by washing the cells in ice-cold medium before filtration.

2 The binding of [^{3}H]-mepyramine sensitive to 2 μM temelastine, measured after 10 min equilibration at 37°C, failed to saturate and was resolved into an hyperbola and an apparently linear component, whereas the fit to the binding of [^{3}H]-mepyramine sensitive to 2 μM pirdonium was not significantly improved over that to an hyperbola. The mean K_d for the binding of [^{3}H]-mepyramine to the saturable component, 2.5 ± 0.4 nM, was in close agreement with the value of 3.5 nM for mepyramine derived from inhibition of histamine H_1-receptor-mediated inositol phosphate formation in U373 MG cells.

3 Curves for the inhibition of the binding of 5 nM [^{3}H]-mepyramine to U373 MG cells by histamine H_1-receptor antagonists were biphasic and were fitted to a two site-model. Affinities calculated from the best-fit IC_{50} values for the high-affinity site correlated well with those expected for binding to H_1-receptors.

4 The percentages of the high-affinity site in curves of the inhibition of [^{3}H]-mepyramine binding to intact U373 MG cells by two tertiary amine antagonists, *nor*pirdonium and 4-methyldiphenhydramine, 68 ± 3 and $63 \pm 4\%$, were significantly greater than the percentages of the high-affinity site in the inhibition curves of their quaternary derivatives, 50 ± 1 and $45 \pm 3\%$, respectively. Similarly, the percentage of the high-affinity site for unlabelled mepyramine, $65 \pm 7\%$, was greater than for the non-cell penetrant H_1-antagonist temelastine, $42 \pm 5\%$.

5 Incubation of U373 MG cells with 100 μM histamine at 37°C, followed by washing twice in ice-cold medium and then incubation with 1-15 nM [^{3}H]-mepyramine for 120 min at 4°C, resulted in a decrease in the binding of [^{3}H]-mepyramine sensitive to 2 μM pirdonium, compared to control cells not exposed to histamine. The binding of [^{3}H]-mepyramine in the absence of pirdonium was not altered by histamine pretreatment, whereas the level of the pirdonium-insensitive binding was significantly increased, except after 1 min exposure to histamine. The decreases in the pirdonium-sensitive binding after 5, 10 and 60 min incubation with 100 μM histamine were 41 ± 6, 56 ± 6 and $67 \pm 8\%$, respectively, but the decrease after 1 min incubation with histamine, $16 \pm 8\%$, was not statistically significant.

6 The results are consistent with the binding of [^{3}H]-mepyramine to intact U373 MG cells being to both plasma membrane and intracellular histamine H_1-receptors. The high-affinity binding sensitive to the non-cell penetrant quaternary compounds and to temelastine is thus to plasma membrane H_1-receptors. On exposure to 100 μM histamine receptors are translocated to the intracellular pool, since the change in the high-affinity binding of [^{3}H]-mepyramine is primarily in the level of the pirdonium-insensitive binding, rather than in the total binding.

Keywords: Histamine H_1-receptors; desensitization; receptor internalisation; [^{3}H]-mepyramine; U373 MG astrocytoma cells; pirdonium

Introduction

Receptor internalisation is well established as a mechanism for the regulation of agonist activity at G protein-coupled receptors, such as those for angiotensin II (see e.g. Hunyady *et al.*, 1994), acetylcholine (Koenig & Edwardson, 1994, and references therein) and noradrenaline (Leeb-Lunberg *et al.*, 1987; Hausdorff *et al.*, 1990). However, although there have been numerous studies of the mechanisms involved in the desensitization of responses to histamine at H_1-receptors in intestinal smooth muscle (Barsoum & Gaddum, 1935; Cantoni & Eastman, 1946; Gosselin & Gosselin, 1973; Hishinuma & Uchida, 1987; 1988; 1991; Horio *et al.*, 1990a,b; Leurs *et al.*, 1990; 1991), brain slices (Quach *et al.*, 1981; Bristow *et al.*, 1993) and cells in culture (Taylor & Richelson, 1979; Brown *et al.*, 1986; Nakahata & Harden, 1987; Dillon-Carter & Chuang, 1989; Cowlen *et al.*, 1990; Smit *et al.*, 1992; Bristow & Zamani, 1993; Dickenson & Hill, 1993; McCreath *et al.*, 1994), a number of which have provided evidence for changes at the level of the receptor (Brown *et al.*, 1986; Nakahata & Harden, 1987; Hishinuma & Uchida, 1988; Cowlen *et al.*, 1990; Horio *et al.*, 1990b; Leurs *et al.*, 1990; Dickenson & Hill, 1993), there is no direct experimental evidence for histamine-induced H_1-receptor internalisation. The problems inherent in measuring binding to intact cells with [^{3}H]-mepyramine, a tertiary amine which will cross the cell membrane and bind to intracellular

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sites and become concentrated in compartments with a low pH, have been noted some years ago (Maloteaux *et al.*, 1983). We have attempted to avoid these difficulties by synthesizing a quaternary amine radioligand for the H_1-receptor, [^{3}H]-(+)-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP), which should bind only to sites on the plasma membrane (Treherne & Young, 1988a). However, attempts to use [^{3}H]-QMDP to label H_1-receptors on intact cells have so far been unsuccessful, and we have therefore set out to develop a protocol with [^{3}H]-mepyramine which would exploit (a) the very slow dissociation of [^{3}H]-mepyramine from H_1-receptors at temperatures below 10°C (Wallace & Young, 1983; Treherne & Young, 1988b), which should allow extensive washing of cells, and (b) the availability of non-penetrant and penetrant H_1-receptor antagonists, which might be used to determine binding to plasma membrane H_1-receptors and plasma membrane + intracellular receptors, respectively. We describe here a study of the characteristics of the binding of [^{3}H]-mepyramine to intact human U373 MG astrocytoma cells, which possess histamine H_1-receptors coupled to the activation of phosphoinositidase C (Arias-Montaño *et al.*, 1994), and the effect on [^{3}H]-mepyramine binding of pre-exposure of the cells to a high concentration of histamine.

Methods

Culture of U373 MG cells

Human U373 MG astrocytoma cells (National Culture Collection, Porton Down) were grown to near confluence in Dulbecco's modified Eagle medium/nutrient mixture F-12 (1:1 v/v; Gibco), containing 10% (v/v) bovine foetal calf serum and 2 mM glutamine (Gibco) and supplemented with penicillin (100 u ml^{-1}), streptomycin (0.1 mg ml^{-1}) and amphotericin B (250 ng 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 once with approximately 10 ml phosphate buffered saline (in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5), pH 7.5, containing 0.6 mM EDTA, before dissociation with 10 ml trypsin/EDTA (500–750 BAEE units ml^{-1}/0.6 mM, Sigma). After centrifugation at 220 *g* for 5 min the cells were resuspended in a *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (HEPES)-buffered medium (in mM: NaCl 120, KCl 5.4, $MgCl_2$ 1.6, $CaCl_2$ 1.8, D-glucose 11, HEPES 25), adjusted to pH 7.4 with NaOH (final concentration of Na^+ 132.6 mM) and kept at 37 °C for 30 min for equilibration. Total cell protein was determined essentially as described by Lowry *et al.* (1951).

Measurement of [^{3}H]-mepyramine binding

Measurements of [^{3}H]-mepyramine binding were made in the HEPES-buffered medium, pH 7.4, containing 1–45 nM [^{3}H]-mepyramine and U373 MG cells (*circa* 3×10^5 cells, approximately 0.3 mg whole cell protein) in the presence or absence of 2 μM temelastine or 2 μM pirdonium (final volume 1 ml). For the determination of curves of the inhibition of the binding of [^{3}H]-mepyramine incubations contained approximately 5 nM [^{3}H]-mepyramine, inhibitor and cells. The actual concentration of [^{3}H]-mepyramine present was determined by counting an aliquot of the [^{3}H]-mepyramine/HEPES medium. In both sets of experiments, incubation was for 10 min at 37°C and was terminated by addition of 3 ml ice-cold buffer and centrifugation at 220 *g* for 5 min. The cells were resuspended in 3 ml ice-cold buffer and the suspension filtered through Whatman GF/B glass fibre filters (pre-soaked for at least 3 h in 0.3% polyethylenimine) using a 24-place cell harvester (Brandel, Gaithersburg, Md, U.S.A.). The filters were transferred to scintillation vial inserts, 3 ml scintillator (Emulsifier Safe, Packard) added and the vials allowed to stand for at least 2 h before determination of tritium by scintillation counting. All determinations were made in quadruplicate.

Measurements of the rate of association of 5 nM [^{3}H]-mepyramine with cells in the presence or absence of 2 μM pirdonium were made as above, except that incubation was from 1 to 30 min at 37°C or 5 to 120 min at 6°C. Dissociation rates at 6°C were determined by equilibrating cells with 5 nM [^{3}H]-mepyramine for 10 min at 37°C, in the presence or absence of 2 μM pirdonium, and then either: (i), diluting 4 fold with ice-cold HEPES medium and filtering after 1–120 min at 6°C, or (ii), as (i), but with the addition of 1 μM unlabelled mepyramine (final concentration) and filtering after 5–120 min at 6°C or (iii), diluting 4 fold with ice-cold HEPES medium, centrifugation at 220 *g* for 5 min, resuspension of the cells in 3 ml ice-cold medium and filtering after 5–120 min at 6°C. For measurement of dissociation at 37°C, cells were equilibrated with 5 nM [^{3}H]-mepyramine for 10 min at 37°C in the presence or absence of 2 μM pirdonium before addition of 1 μM unlabelled mepyramine (final concentration). After 5 s–20 min, the mixture was diluted 4 fold with ice-cold HEPES medium and centrifuged at 220 *g* for 5 min. The cells were resuspended in 3 ml ice-cold medium and filtered as above.

Measurement of histamine-induced H_1-receptor internalisation

U373 MG cells (*circa* 7×10^6 cells ml^{-1}) were incubated with 100 μM histamine or with no addition (control) for 1 to 60 min at 37°C and then diluted 4.75 fold with ice-cold HEPES medium and centrifuged at 220 *g* for 5 min. The cells were washed by twice resuspending in 20 ml ice-cold medium and centrifuging at 220 *g* for 5 min, before being resuspended in 4.5 ml ice-cold medium. The protein content of each cell suspension was determined at this stage. Aliquots (100 μl) of control and histamine-pretreated cells suspensions were then incubated with 1–15 nM [^{3}H]-mepyramine in the presence or absence of 2 μM pirdonium for 120 min at 4°C and bound tritium determined as described above.

Inhibition of histamine-induced inositol phosphate accumulation

Measurement and analysis of the inhibition of histamine-induced [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation by 100 nM (±)-QMDP in U373 MG cells prelabelled with [^{3}H]-inositol was carried out as described previously (Arias-Montaño *et al.*, 1994).

Analysis of data

Curves of the temelastine-sensitive binding of [^{3}H]-mepyramine *versus* the concentration of [^{3}H]-mepyramine were fitted to a hyperbola + a linear component using the Harwell Library non-linear regression programme VB01A. The actual equation fitted was:

$$[^3\text{H}]\text{-mepyramine bound} = B_{max}L/(L + K_d) + m.L$$

where L is the concentration of [^{3}H]-mepyramine, m is the slope of the linear component, and B_{max} and K_d are the parameters characterizing the hyperbolic component. Binding curves defined by 2 μM pirdonium were fitted to an hyperbola (m = 0 in the equation above). Each point was weighted according to the reciprocal of the variance associated with it. In the experiments in which internalisation of [^{3}H]-mepyramine binding sites was measured, the proportion of H_1-receptors internalised was calculated from the ratio of the best-fit values of B_{max} for the pirdonium-sensitive binding of [^{3}H]-mepyramine to pretreated and control cells, after adjustment for the relative amounts of cell protein present in the incubation with [^{3}H]-mepyramine.

Curves of the inhibition of the binding of [^{3}H]-mepyramine were fitted to a two-site (double-hyperbola) model:

$$\%\text{ of uninhibited binding of } [^3\text{H}]\text{-mepyramine} = 100 - N_1.I/(I + IC_{50.1}) - N_2.I/(I + IC_{50.2})$$

where I is the concentration of the inhibitor, N_1 and N_2 are the percentages of the high and low affinity sites, respectively, and $IC_{50.1}$ and $IC_{50.2}$ are the concentrations of inhibitor producing 50% inhibition at sites 1 and 2, respectively. The data were weighted and fitted as above. The dissociation constant of the inhibitor for the high affinity site, K_i, was calculated from the relationship $K_i = IC_{50.1}/([^3H\text{-mepyramine}]/K_d + 1)$, where $K_{-\,>d}$ is the dissociation constant for [^{3}H]-mepyramine. In the special case of mepyramine *versus* [^{3}H]-mepyramine this relationship simplifies to $K_d = IC_{50.1} - [^3H\text{-mepyramine}]$, assuming that the substitution of one atom of tritium for hydrogen makes no measurable difference to K_d for mepyramine.

Data for association and dissociation of [^{3}H]-mepyramine were fitted assuming a reversible interaction with a single set of binding sites. The equation fitted for association was $B = B_{equil}(1\text{-}e^{k-obs.t})$, where B is the amount of [^{3}H]-mepyramine bound at time t, B_{equil} the amount bound at equilibrium, and k_{obs} the observed association rate constant. For dissociation the equation was $B = B_o.e^{-k.t}$, where B_o is the amount bound at time zero and k is the dissociation rate constant from the H_1-receptor.

Statistical comparison of the goodness of fit to two models (one-site *versus* two-site inhibition curves or an hyperbola *versus* an hyperbola + linear component for binding curves) was made by testing the significance of the excess sum of squares:

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

where SS_1 and SS_2 are the residual sums of squares for the simpler and more complex model, respectively, with df_1 and df_2 degrees of freedom.

In experiments on the effect of pre-exposure of cells to histamine on the total and pirdonium-sensitive binding of [^{3}H]-mepyramine, in which experimental limitations made it impractical to obtain a sufficient number and spread of data points to allow accurate statistical analysis of differences in the position of curves via curve fitting with and without shared parameters, a simpler statistical test was employed. The probability that the amount of [^{3}H]-mepyramine bound at all the 5 concentrations of [^{3}H]-mepyramine used in each experiment is less (or more) after histamine pretreatment than in control cells is 0.031. If only 4 of the 5 values are lower (or greater), then the difference between the curves is not significant ($P > 0.05$).

Drugs

[*Pyridinyl*-5-^{3}H]-mepyramine, 27 Ci mmol^{-1}, was obtained from Amersham International. *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (HEPES) and histamine dihydrochloride were purchased from Sigma. Temelastine was a gift from Smith, Kline & French Research Ltd, and 4-methyldiphenhydramine, *nor*pirdonium and pirdonium were kindly provided by Prof. H. Timmerman, Department of Pharmacochemistry, Free University of Amsterdam. (±)-N-methyl-4-methyldiphenhydramine (±-QMDP) was synthesized by Dr D.H. Marrian. The structures of the antagonists used are given elsewhere (Gibson *et al.*, 1994).

Results

Binding of [^{3}H]-mepyramine to intact U373 MG cells

The rate of formation of the complex between 5 nM [^{3}H]-mepyramine and a plasma membrane binding site on intact human U373 MG astrocytoma cells, as defined by the binding of [^{3}H]-mepyramine sensitive to 2 μM pirdonium, a quaternary histamine H_1-receptor antagonist, was sensitive to the temperature of the incubation. At 37°C the binding of 5 nM [^{3}H]-mepyramine was rapid (Figure 1a), with a half-time of 0.9 ± 0.4 min, as determined by fitting a single exponential to the association data (3 determinations). However, at 4°C the pirdonium-sensitive binding of 5 nM [^{3}H]-mepyramine only approached a plateau level after 90–120 min (Figure 1b), with a mean best-fit half-time of 19 ± 3 min (3).

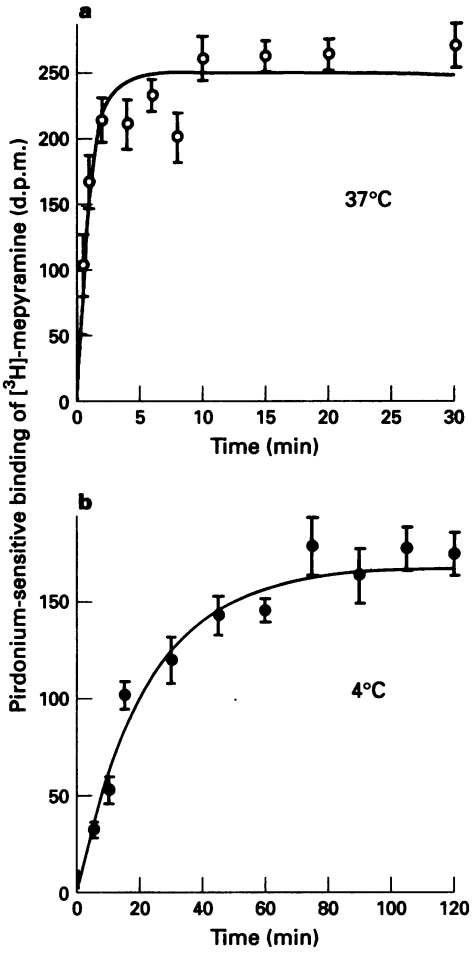

Figure 1 Rate of association of [^{3}H]-mepyramine with H_1-receptors on intact U373 MG cells at (a) 37°C and (b) 4°C. Measurements of the association of the binding of [^{3}H]-mepyramine to U373 MG cells in the presence or absence of 2 μM pirdonium were made as described under Methods. The points represent the pirdonium-sensitive binding ± s.e.mean of 4.8 nM (a) or 5.12 nM (b) [^{3}H]-mepyramine from quadruplicate determinations within a single experiment, which was repeated twice further at each temperature with similar results. The curves drawn are the best-fit lines to a single exponential. (a) 37°C. Best fit values ± estimated error: k_{on}, the observed on-rate constant, 1.01 ± 0.23 min^{-1}, B_{max} 250 ± 8 d.p.m.. (b) 4°C. k_{on} 0.046 ± 0.006 min^{-1}, B_{max} 168 ± 8 d.p.m.

The rate of dissociation of the pirdonium-sensitive binding of [^{3}H]-mepyramine from U373 MG cells was particularly temperature-dependent, as we have reported previously for binding to H_1-receptors in membrane preparations (Wallace & Young, 1983; Treherne & Young, 1988b). At 37°C dissociation induced by the addition 1 μM unlabelled mepyramine was rapid, with an estimated $t_{1/2}$ of 1.5 ± 0.3 min (weighted mean of the best-fit values from 2 independent experiments, data not shown). However, at 6°C there was negligible dissociation over a period of 120 min after dilution of the incubation mixture at 37°C with a three fold excess of ice-cold medium (Figure 2a). Addition of 1 μM mepyramine at the dilution stage failed to increase the rate of dissociation (3 measurements, data not shown). We have taken advantage of this very slow dissociation to reduce the level of pirdonium-insensitive binding of [^{3}H]-mepyramine to the cells (56 ± 5%, 6 measurements) by centrifuging the incubation mixture immediately after dilution with ice-cold buffer and then resuspending the cells in 3 ml ice-cold medium before filtration. There was no apparent change in the amount of bound [^{3}H]-mepyramine when the period between resuspension and filtration was varied between 0 and

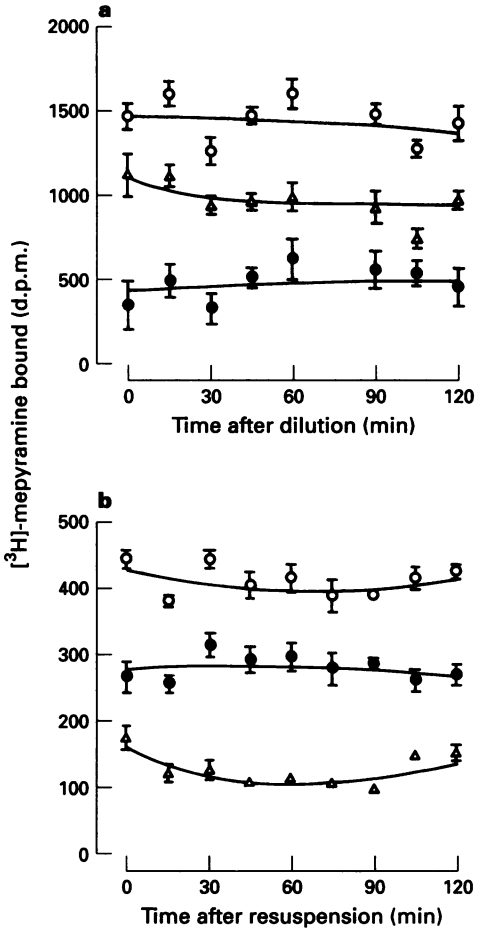

Figure 2 Dissociation of bound [^{3}H]-mepyramine from U373 MG cells at 6°C. (a) Cells labelled with 5 nM [^{3}H]-mepyramine at 37°C for 10 min were diluted 4 fold with ice-cold HEPES buffer and incubation continued at 6°C for 0–120 min before filtration. (b) Cells were labelled and diluted as in (a), but washed by centrifugation and resuspension in ice-cold HEPES medium before filtration after 0–120 min at 6°C. The data in (a) and (b) are means ± s.e.mean from quadruplicate determinations within a single experiment, which was repeated twice further for each protocol with similar results. Where no error bars are shown the error was within the size of the symbol. The lines have been drawn by inspection. (○) Binding in the absence of pirdonium; (△) binding in the presence of 2 μM pirdonium; (●) pirdonium-sensitive binding.

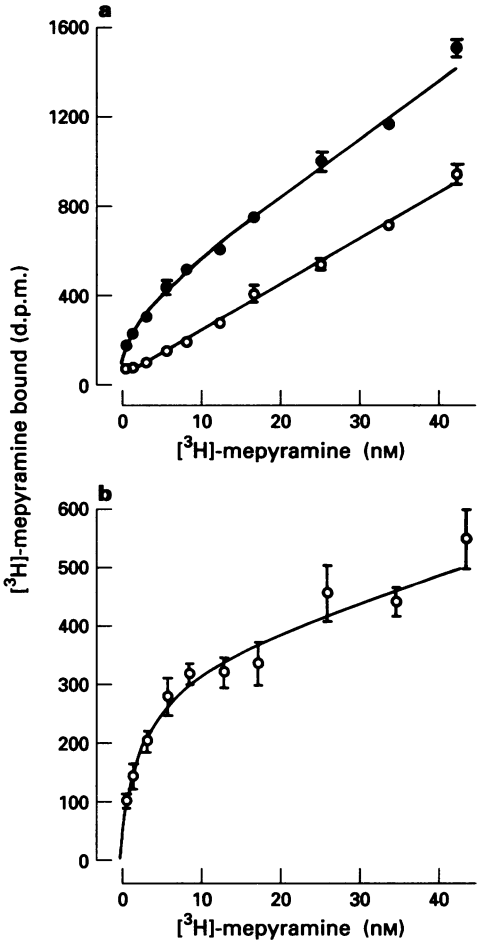

Figure 3 Concentration-dependence of the binding of [^{3}H]-mepyramine to U373 MG cells. Incubations with [^{3}H]-mepyramine in the presence or absence of 2 μM temelastine were for 10 min at 37°C. (a) Points are the means ± s.e.mean of quadruplicate determinations within a single experiment. Where no error bars are shown the error was within the size of the symbol. The lines have been drawn by inspection. (●) Binding in the absence of temelastine; (○) binding in the presence of 2 μM temelastine. (b) Temelastine-sensitive binding. The curve drawn is the best-fit line for an hyperbola + a linear component (see Methods and text). Best-fit values ± estimated errors: K_d 1.6 ± 0.4 nM; B_{max} 10 ± 1 fmol mg^{-1} cell protein, slope of the linear component 4.6 ± 1.2 × 10^{10} d.p.m. M^{-1}. The whole experiment was repeated on 3 further occasions.

120 min (Figure 2b), but the mean level of pirdonium-insensitive binding was reduced to 39 ± 5% (3). This protocol was used routinely in further experiments.

Measurements of the equilibrium binding of [^{3}H]-mepyramine to U373 cells after 10 min incubation at 37°C were made in the presence or absence of 2 μM pirdonium or 2 μM temelastine. In experiments with temelastine, the inhibitor-sensitive binding consistently failed to approach saturation, even at 40–50 nM [^{3}H]-mepyramine and in each of 4 experiments the data points were fitted significantly better by an hyperbola + a linear component (e.g. Figure 3b) (mean best-fit value of the slope of the linear component 5.7 ± 0.5 × 10^9 d.p.m. M^{-1}). The linear component presumably represents the foot of a binding curve or curves to lower affinity site(s) for [^{3}H]-mepyramine sensitive to temelastine. There was some indication of the presence of a linear component in one of three experiments using 2 μM pirdonium, but in no case was the fit to an hyperbola + a linear component significantly better than that to an hyperbola alone. The mean best-fit values of K_d for the hyperbolic component from experiments using temelastine and for the fitted hyperbola with pirdonium were similar, 1.9 ± 0.3 nM (4) and 3.2 ± 0.5 nM (3) and both were in good agreement with the values of the K_d for mepyramine binding to the histamine H_1-receptor in U373 MG cells determined from inhibition of [^{3}H]-mepyramine binding to U373 MG cell membranes, 2.5 ± 1.0 nM (Arias-Montaño *et al.*, 1994) and from inhibition of histamine-induced [^{3}H]-inositol monophosphate accumulation, 3.5 ± 0.3 nM (Arias-Montaño *et al.*, 1994). The mean maximum binding of [^{3}H]-mepyramine to the saturable component in intact cells, 14 ± 3 and 11 ± 1 fmol mg^{-1} cell protein, defined by pirdonium and temelastine, respectively, was comparable with the 86 ± 19 fmol mg^{-1} membrane protein measured for U373 MG cell membranes (Arias-Montaño *et al.*, 1994).

Inhibition of the binding of [^{3}H]-mepyramine to U373 MG cells

The curve for the inhibition of the binding of 5 nM [^{3}H]-mepyramine to U373 MG cells by unlabelled mepyramine was biphasic (Figure 4), but the proportion of the high affinity site, mean 65 ± 7% (3), was greater than that observed in inhibition curves for temelastine, 42 ± 5% (3). This suggested that whereas [^{3}H]-mepyramine and unlabelled mepyamine can cross the cell membrane and bind to receptors both on the plasma membrane of the cells and on internal membranes, temelastine

was not cell penetrant, consistent with its inability to cross the blood-brain barrier (Calcutt *et al.*, 1987), and was binding only to H_1-receptors on the plasma membrane. We have tested this proposition by comparing the relative extents to which two tertiary amines and their methylated, quaternary, derivatives were able to inhibit the binding of [^{3}H]-mepyramine to the intact cells. Representative examples of inhibition curves for *nor*pirdonium and for the quaternary derivative, pirdonium are shown in Figure 5. The points have been fitted to a double hyperbola in each case, but the striking feature is that the proportion of the high-affinity site is lower for the quaternary compound. The inhibition curves for 4-methyldiphenhydramine and its quaternary analogue, N-methyl-4-methyldiphenhydramine (QMDP) showed a similar difference. In every experiment with inhibitors, with the sole exception of one experiment with pirdonium, the fit of the inhibition curve to a two-site model was significantly better than to a single site.

The proportions of the high affinity site in the inhibition curves of the two pairs of tertiary/quaternary amines observed in 3 experiments with each compound are set out in Table 1. A fourth experiment with pirdonium, which had the lowest proportion of the high-affinity component, 48±2%, has been omitted in order to maintain a balanced experimental design. Analysis of the data by two-way analysis of variance showed the difference between the two groups (tertiaries *v.* quaternaries) to be highly significant ($F=41.73$ with 1,9 degrees of freedom; $P<0.001$), whereas there was no significant difference within groups (i.e. between *nor*pirdonium and 4-methyldiphendhyramine or between pirdonium and QMDP) ($F=3.36$ with 1, 9 degrees of freedom). Inclusion of mepyramine and temelastine in the statistical analysis (as cell penetrant and non-penetrant, respectively) led to the same conclusion (between groups $F=37.83$, 1, 14 d.f., $P<0.001$; within groups $F=1.16$, 2, 14 d.f., NS).

The dissociation constants of the inhibitors calculated from the best-fit values of the IC_{50} for the high-affinity site from the individual inhibition curves are set out in Table 2. The values are similar to those determined from inhibition of histamine-induced [^{3}H]-IP_1 formation in human HeLa cells (Arias-Montaño & Young, 1993) and from inhibition of the binding of [^{3}H]-QMDP to guinea-pig cerebellar membranes or inhibition of histamine-induced contraction of the longitudinal muscle from guinea-pig small intestine (Treherne & Young, 1988a). Measurements were also made of the inhibition of histamine-induced [^{3}H]-IP_1 formation in U373 MG cells by 100 nM QMDP, but in 2 of 4 experiments constraining the concentration-response curves for histamine in the presence and absence of QMDP to have a common Hill coefficient and maximum response, as required for a competitive antagonist, significantly worsened the fit to the data. However, the estimate of the K_d for QMDP from the combined data, 8.4±3.4 nM (4), was in very good agreement with the value from inhibition of [^{3}H]-mepyramine binding, 8.2±2.3 nM

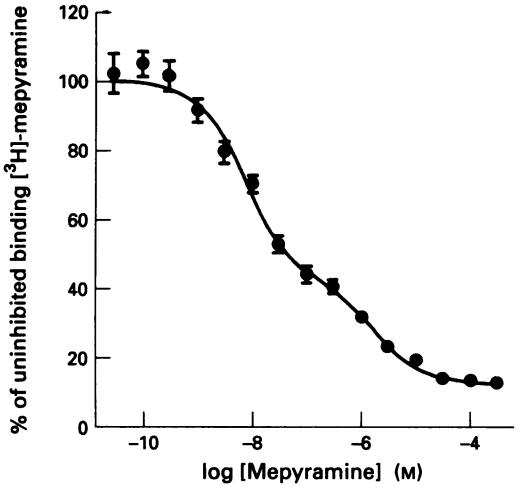

Figure 4 Inhibition of the binding of 4.5 nM [^{3}H]-mepyramine to U373 MG cells by unlabelled mepyramine. The points are means ±approximate s.e.mean from quadruplicate determinations within a single experiment. Where no error bars are shown the error was within the size of the symbol. The binding in the absence of unlabelled mepyramine was 449±11 d.p.m. (12). The curve drawn is the best-fit line to a two-site inhibition model (see Methods). Best-fit values±estimated error: percentages of the high- and low-affinity sites 58±2 and 28±2%, respectively, apparent K_d values 7.9±1.4 nM and 1.9± 0.3 μM.

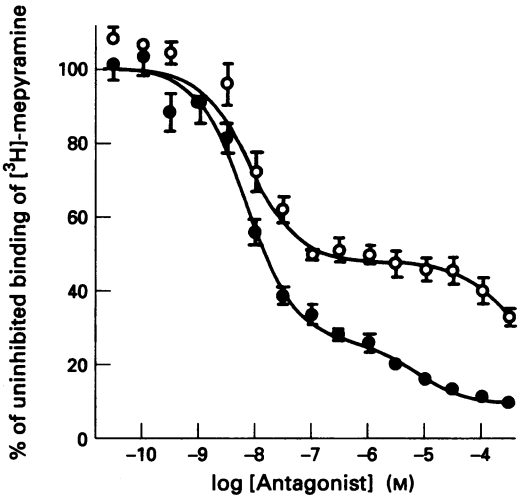

Figure 5 Inhibition by *nor*pirdonium and pirdonium of the binding of [^{3}H]-mepyramine to U373 MG cells. The points are the means±approximate s.e.mean of quadruplicate determination from independent experiments with each antagonist. Where no error bars are shown the error was within the size of the symbol. The binding in the absence of inhibitor was 301±7 d.p.m. (12) in the experiment with *nor*pirdonium (4.8 nM [^{3}H]-mepyramine) and 365±12 d.p.m. (11) with pirdonium (4.7 nM [^{3}H]-mepyramine). The curves drawn are the best-fit lines to a two-site inhibition model (see Methods). Best-fit values±estimated error: percentages of high- and low-affinity sites for pirdonium 52±2 and 28±4%, respectively, apparent K_d values 7.7±2.8 nM and 0.3±0.8 mM: for *nor*pirdonium the corresponding values were 73±1 and 17±1% and 6.9±1.0 nM and 6.4±1.9 μM. (●) *Nor*pirdonium; (○) pirdonium.

Table 1 Percentage of the high affinity site from fitting curves of inhibition of [^{3}H]-mepyramine binding to intact U373 MG cells to a two-site model

	% of high-affinity site				
Cell-penetrant compounds			*Non-penetrant compounds*		
Norpirdonium			Pirdonium		
73.4	65.1	64.8	48.0	49.9	52.1
	68±3			**50±1**	
4-Methyldiphenhydramine			QMDP		
67.9	54.6	65.4	45.1	40.4	49.9
	63±4			**45±3**	
Mepyramine			Temelastine		
57.8	59.9	78.2	40.4	51.3	34.2
	65±7			**42±5**	

Values are the percentages of the high-affinity site from fitting curves of inhibition of the binding of [^{3}H]-mepyramine (e.g. Figures 3 and 4) to a two-site model (see Methods). The figures in bold type are the means±s.e.mean for each compound. The difference in the percentages of the high-affinity component between presumed cell-penetrant and non-penetrant compounds was highly significant ($P<0.001$), whereas the difference within groups was non-significant ($P>0.05$, two-way analysis of variance). The significance is the same whether mepyramine/temelastine are included or not.

(Table 2). This is consistent with the high affinity component of the binding of [^{3}H]-mepyramine to U373 MG cells being to the histamine H_1-receptor.

Histamine-induced H_1-receptor internalisation

To investigate histamine-induced H_1-receptor internalisation, U373 MG cells were exposed to 100 μM histamine for 1, 5, 10 or 60 min at 37°C before dilution 4.75 fold with ice-cold medium and washing by twice centrifuging and resuspending in ice-cold HEPES medium. The cell suspension was then incubated with 1–15 nM [^{3}H]-mepyramine at 4°C for 120 min in the presence or absence of 2 μM pirdonium and bound [^{3}H]-mepyramine determined as described above. The results of two experiments in which cells were treated with 100 μM histamine for 1 min or 10 min are shown in Figure 6. In neither experiment was there any significant change in the total binding of [^{3}H]-mepyramine (i.e. in the absence of 2 μM pirdonium) compared with control cells taken through the same procedure, but with no added histamine. However, it is striking that after 10 min pretreatment with histamine there has been a significant increase in the level of pirdonium-insensitive binding of [^{3}H]-mepyramine (Figure 6b). This pattern was observed in all experiments with 5, 10 or 60 min histamine pretreatment. In only 2 of the 9 experiments was there any significant decrease in the total binding of [^{3}H]-mepyramine after exposure to histamine, whereas the increase in the pirdonium-sensitive binding was significant in all but 1 of the 9 experiments. This is consistent with a movement of receptors from the plasma membrane to an intracellular locus, where they are still accessible to [^{3}H]-mepyramine, but not accessible to the quaternary antagonist, pirdonium. However, 1 min pretreatment of cells with 100 μM histamine did not lead to any significant change in either total or pirdonium-sensitive binding of [^{3}H]-mepyramine in any of the 3 experiments (e.g. Figure 6a).

The limitation on the number of concentrations of [^{3}H]-mepyramine which could be employed in each of these experiments made it impractical to obtain sufficient data points to test whether the fit of the pirdonium-sensitive binding to an hyperbola + linear component was significantly better than to an hyperbola alone. We have therefore obtained an unbiased estimate of the change in the plasma membrane H_1-receptor binding by limiting the maximum concentration of [^{3}H]-mepyramine used to approximately 15 nM and then fitting an

Table 2 Dissociation constants for antagonists calculated from inhibition of the binding of [^{3}H]-mepyramine to the high-affinity site in intact U373 MG cells

Inhibitor	K_d (nM) *U373 MG cells*	*HeLa cells**	*Guinea-pig†*
Mepyramine	5.5±2.3	3.4	0.9
Temelastine	1.1±0.7	0.7	0.8
*Nor*pirdonium	2.0±0.5	–	1.8
Pirdonium	2.7±0.7	–	1.0
4-Methyldiphenhydramine	2.9±0.9	–	2.2
QMDP	8.2±2.3	14.0	2.5

K_d values for intact U373 MG cells have been calculated from the best-fit IC_{50} values for inhibition of the binding of 3.1–4.8 nM [^{3}H]-mepyramine to the high-affinity site, obtained from fitting a two-site model to the inhibition data (see Methods). Values are the means±s.e mean from 3 determinations.

*Determined from inhibition of histamine-induced [^{3}H]-IP_1 formation in human HeLa cells (taken from Arias-Montaño & Young, 1993).

†Determined from inhibition of the binding of [^{3}H]-(+)-QMDP to guinea-pig cerebellar membranes or (for *nor*pirdonium and pirdonium) from inhibition of histamine-induced contraction of the longitudinal muscle from guinea-pig small intestine (taken from Treherne & Young, 1988a).

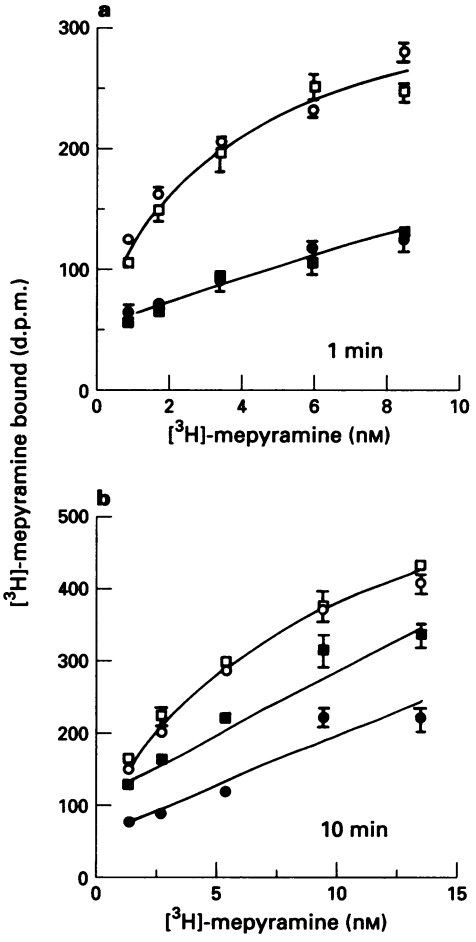

Figure 6 Effect of pretreatment with histamine on the pirdonium-sensitive binding of [^{3}H]-mepyramine to U373 MG cells. Cells were pretreated with 100 μM histamine at 37°C for (a) 1 min or (b) 10 min, washed in ice-cold medium, and the binding of [^{3}H]-mepyramine measured at 4°C in the absence (○, □) or presence (●, ■) of 2 μM pirdonium as described under Methods. The data in each panel are from a single experiment and are the mean±s.e.mean from quadruplicate determinations. Where no error bars are shown the error was within the size of the symbol. The lines have been drawn by inspection. (○, ●) Control cells; (□, ■) cells pretreated with 100 μM histamine.

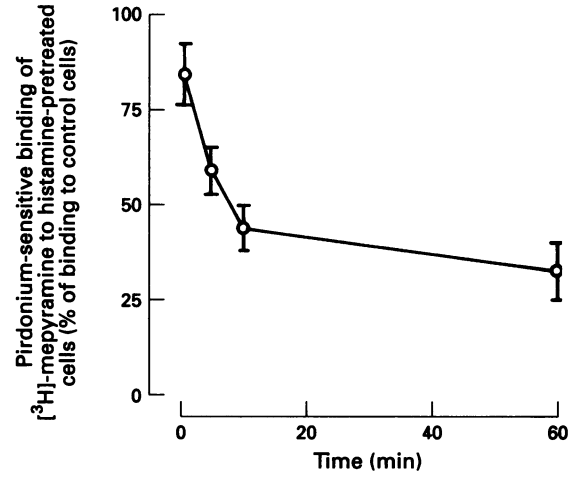

Figure 7 Histamine-induced H_1-receptor internalisation. The change in the number of plasma membrane receptors, expressed as a percentage of the value in control cells, was calculated by fitting an hyperbola to the pirdonium-sensitive binding obtained from experiments such as those shown in Figure 6 (see text and Methods) and, hence, obtaining an estimate of B_{max}. Each point is the weighted mean±approximate s.e.mean of the percentages from 3 experiments.

hyperbola to the pirdonium-sensitive binding in control and histamine-pretreated cells. The best-fit values of B_{max} were corrected for any difference in the protein content of the final suspensions of control and histamine-pretreated cells. The mean change in plasma membrane H_1-receptor binding in histamine-pretreated cells, expressed as a percentage of that in control cells as a function of time (3 determinations at each time), is shown in Figure 7. After exposure to 100 μM histamine for 1 min the degree of H_1-receptor internalisation is small and statistically non-significant, but by 10 min the agonist-induced movement into the intracellular compartment is essentially complete.

Discussion

The potential difficulties in using cell-permeable radioligands for the measurement of binding to cell surface receptors were documented some years ago (Maloteaux *et al.*, 1983) and initially we set out to circumvent them by synthesizing a non-penetrant radioligand for the histamine H_1-receptor, [^{3}H]-QMDP, a quaternary amine, (Treherne & Young, 1988a), in analogy to the use of [^{3}H]-N-methylscopolamine (Galper *et al.*, 1982) and CGP-12177 (Hertel *et al.*, 1983a) to label plasma membrane muscarinic and β-adrenoceptors, respectively. However, attempts to label H_1-receptors on U373 MG astrocytoma cells with [^{3}H]-QMDP have to date been unsuccessful. Large amounts of binding sensitive to mepyramine, doxepin and temelastine can be detected, but the parameters characterizing this binding bear no relationship to those expected for binding to the H_1-receptor (S.H. & J.M.Y., unpublished observations). The lack of specificity of temelastine, which appeared to have a low affinity for secondary sites labelled by [^{3}H]-QMDP in guinea-pig cerebellum (Treherne & Young, 1988a), is disappointing, but there are clearly also temelastine-sensitive, non-H_1-receptor binding sites for [^{3}H]-mepyramine on U373 MG cells (Figure 3b). The propensity of [^{3}H]-mepyramine to bind to medium-affinity sites with no obvious relationship to the H_1-receptor has been apparent from early studies on peripheral tissues (Chang *et al.*, 1979; Hill & Young, 1981), and such sites are present, although not always recognised as such, in the DDT_1MF_2 (Mitsuhashi & Payan, 1988), HeLa (Raymond *et al.*, 1991; Arias-Montaño & Young, 1993) and P19 (Bloemers *et al.*, 1993) cell lines. Rat liver membranes are particularly rich in a high-affinity, non-H_1-receptor binding site for [^{3}H]-mepyramine (Leurs *et al.*, 1989), which appears to be a member of the cytochrome P-450 family (Liu *et al.*, 1992). However, this site was not evident in brain membranes (Liu *et al.*, 1992) and the good correlation between the dissociation constants of antagonists determined from inhibition of the high-affinity binding of [^{3}H]-mepyramine to U373 MG astrocytoma cells and from functional responses in intact tissues (Table 2) argues strongly that the high-affinity binding is to histamine H_1-receptors. The very good agreement between the K_d values for mepyramine determined from the temelastine- and pirdonium-sensitive binding of [^{3}H]-mepyramine to the cells, 1.9 and 3.2 nM (overall mean K_d 2.5$\pm$0.4 nM), and from inhibition of histamine-stimulated inositol phosphate formation in the same cells, 3.5 nM (Arias-Montaño *et al.*, 1994), lends further support to this proposition, as does the good correlation between K_d values for QMDP from inhibition of binding and response.

The relatively high proportion of the high-affinity site in inhibition curves of mepyramine *versus* [^{3}H]-mepyramine, 65$\pm$7%, makes it clear that binding to other intracellular sites or accumulation in compartments of low pH is not a severe problem in U373 MG cells. This has allowed the determination of the proportion of receptors in the plasma membrane and, from the difference of the proportion of high-affinity site between a permeant and a non-permeant inhibitor, in intracellular locations. This is demonstrated most clearly, and with high statistical significance, by the comparison between the proportion of [^{3}H]-mepyramine binding which could be inhibited with high affinity by two tertiary amine inhibitors and by their quaternary derivatives (Table 1). It also indicates that temelastine is non-cell-penetrant, as might have been predicted from its limited ability to enter the CNS (Calcutt *et al.*, 1987). This, in turn, suggests that the degree to which an H_1-antagonist inhibits the binding of [^{3}H]-mepyramine to intact cells compared to standard permeant/impermeant compounds, should, in principle, serve as a relatively simple *in vitro* assay to determine the likelihood of the ability of the compound to penetrate the CNS and hence produce sedative side-effects in man.

The low level of H_1-receptor binding of [^{3}H]-mepyramine to the washed U373 MG cells (see the number of d.p.m. on the ordinates of Figures 1, 2b and 6) limits the accuracy with which curves of inhibition of [^{3}H]-mepyramine binding can be determined, but there is no indication in the curves for the cell-penetrant inhibitors that there is any difference in their affinities for intracellular and plasma membrane H_1-receptors. A similar situation exists for the binding of most, but not all, antagonists to the two populations of β-adrenoceptors in C6-glioma cells (Hertel *et al.*, 1984). The particular advantage of the absence of any obvious change in affinity of [^{3}H]-mepyramine is that any movement of receptors between the plasma membrane and intracellular sites would not be expected to have any significant effect on the total binding of [^{3}H]-mepyramine. This is indeed observed in the experiments designed to measure histamine-induced H_1-receptor internalisation, in that after 5 min or more exposure to 100 μM histamine there is no significant change in the total binding of [^{3}H]-mepyramine, but the proportion of the binding sensitive to the cell-impermeant inhibitor, pirdonium, is decreased. The lack of change in the total binding argues strongly against the reduction in the pirdonium-sensitive component being due to the persistence of histamine at its binding site and is exactly as predicted if receptors on the plasma membrane have been translocated into the cell. It also indicates that even after 60 min any net down-regulation of H_1-receptors is at most very limited. The time-course and extent of the histamine-induced internalisation of the H_1-receptors on U373 MG cells is closely similar to those reported from studies on α_1-adrenoceptors (Leeb-Lunberg *et al.*, 1987), β-adrenoceptors (Hertel *et al.*, 1983a; Waldo *et al.*, 1983) and muscarinic receptors (Koenig & Edwardson, 1994, and references therein).

The effect of preincubation of intact tissues with histamine on the subsequent binding of [^{3}H]-mepyramine to membranes prepared from the tissues had been measured in three previous studies. In two of these, with longitudinal muscle strips from guinea-pig jejunum (pre-exposed to 100 μM histamine for 15 min; Leurs *et al.*, 1991) and cross-chopped slices of guinea-pig cerebral cortex (100 μM histamine for 30 min; Bristow *et al.*, 1993), no changes were detected in the K_d or B_{max} of the subsequent binding of [^{3}H]-mepyramine (Leurs *et al.*, 1991) or in the parameters of curves of inhibition of [^{3}H]-mepyramine binding by mepyramine and histamine (Bristow *et al.*, 1993). In the third, with cross-chopped slices from mouse cerebral cortex (50 μM histamine for 20 min; Quach *et al.*, 1981), there was a 21% decrease in B_{max}, without any change in K_d. However, in all of these studies the membranes were equilibrated with [^{3}H]-mepyramine for 20–35 min at 37°C or 60 min at 30°C and it seems very likely that under these conditions receptors which had been internalised, but not degraded, would have largely recycled to the plasma membrane. We have made a preliminary attempt to measure the rate of recycling of H_1-receptors back to the plasma membrane in U373 MG cells by pretreating with histamine at 37°C, washing at 4°C and then incubating at 37°C for various periods of time before measurement of [^{3}H]-mepyramine binding at 4°C. However, under these conditions the binding in control cells decreased with time during the second incubation at 37°C, introducing some uncertainty into the measurements of recovery. The indication is that the rate of recovery is probably of the same order as reported for muscarinic receptors, i.e. recovery is complete by 30–60 min (Koenig & Edwardson, 1994).

The evidence of the present study is that the characteristics of histamine-induced internalisation of H_1-receptors are closely similar to those reported for other G protein-coupled receptors. It has been reported that receptor trafficking in murine N1E-115 neuroblastoma cells is markedly reduced in cell suspensions compared to adherent monolayers (Kanba *et al.*, 1990) and the same appears to hold for murine NG 108-115 neuroblastoma cells (Koenig & Edwardson, 1994). However, this did not appear to be a problem in an earlier study with dissociated N1E-115 cells (Cioffi & El-Fakahany, 1989), in which the time-course of loss of [^{3}H]-N-methylscopolamine binding to cell surface receptors was closely similar to that we report here for internalisation of H_1-receptors in U373 MG cells. We have shown previously that histamine induces a marked formation of inositol phosphates in dissociated U373 MG cells (Arias-Montaño *et al.*, 1994) and we have observed that the extent of the response, expressed as a percentage of the basal response, was similar whether measured on dissociated cells or on adherent monolayers (Arias-Montaño & J.M.Y., unpublished observations). We have also checked that cells taken through the centrifugation and resuspension steps in the protocol for the measurement of internalisation (see Methods) still show histamine-induced [^{3}H]-IP_1 accumulation. However, whether the dissociated cells behave in the same way in every respect as a monolayer remains to be established. It is notable that on the evidence of the relative extent of the inhibition of [^{3}H]-mepyramine binding by tertiary and quaternary amines, *circa* 30% of H_1-receptors in unstimulated U373 MG cells appear to be intracellular, in contrast to the much smaller percentage, *circa* 5–19% maximum, of β-adrenoceptor or muscarinic receptors in an intracellular locus in unstimulated monolayers of human 1321N1 astrocytoma (Waldo *et al.*, 1983; Harden *et al.*, 1985) or murine C6-glioma (Hertel *et al.*, 1983b; 1984) cells or in cultured embryo chick heart cells (Galper *et al.*, 1982). Comparative measurements on U373 MG cell monolayers should indicate whether there is an anomaly in the dissociated cells.

The main conclusion to be drawn from the results presented here is that internalisation is a mechanism available for the regulation of H_1-receptor function, at least in astrocytoma cells. Whether it plays any significant role in the rapid desensitization of responses to histamine remains to be established. In the case of the β-adrenoceptor, arguments have been made that the contribution of receptor sequestration to rapid desensitization is small compared to uncoupling of receptor and effector as a consequence of receptor phosphorylation via protein kinase A or β-adrenoceptor kinase (Lohse *et al.*, 1990; Hausdorff *et al.*, 1990). With receptors coupled to phosphoinositidase C (PIC) there is the added complication of a change in the Ca^{2+}-dependence of 1,4,5-IP_3 formation, which occurs within 1 min of stimulation by carbachol of muscarinic receptors in SH-SY5Y neuroblastoma cells (Wojcikiewicz *et al.*, 1994), and which may indicate a change in the state of PIC. The suggestion has been made for type-1 angiotensin II (AII_1) receptors in bovine adrenal glomerulosa cells that there may be a close connection between receptor internalisation and the maintenance of the secondary, Ca^{2+}-dependent phase of 1,4,5-IP_3 formation (Hunyady *et al.*, 1991). However, there are clearly differences between internalisation of H_1-receptors and AII_1 receptors, not least since the lack of significant change in the total binding of [^{3}H]-mepyramine indicates that histamine, unlike AII, is not carried into the cells with the H_1-receptor. Exactly what place internalisation has in the control of histamine H_1-receptor function will require a much more detailed investigation.

We are grateful for generous financial support from the Uchida-Koujirou Memorial Foundation, Japan.

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(Received June 8, 1995
Revised July 18, 1995
Accepted July 21, 1995)