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Thiomersal enhances the binding of histamine to the H_1 receptor, but not histamine-stimulated inositol phosphate formation

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Abstract

Thiomersal (thimerosal) was a weak inhibitor of the binding of [^{3}H]mepyramine to histamine H_1 receptors in guinea-pig cerebellar membranes (11 $\pm$ 1% inhibition at 10 μM, 32 $\pm$ 3% inhibition at 300 μM). However, in the concentration range 3–30 μM, thiomersal enhanced the binding of histamine to the H_1 receptor, as reflected by the displacement of curves of histamine inhibition of [^{3}H]mepyramine binding to lower concentrations, without any change in the Hill coefficient. The ratio of the IC50 values (the concentration giving 50% inhibition) in the absence and presence of thiomersal increased from 1.8 with 3 μM to 3.6 with 30 μM thiomersal. There was no consistent effect of thiomersal at concentrations of 30 μM and below on curves of mepyramine inhibition of [^{3}H]mepyramine binding. In the presence of 10 μM thiomersal histamine-induced accumulation of inositol phosphates in U373 MG astrocytoma cells was partially inhibited (37 $\pm$ 8% inhibition of the maximum response), without any significant change in the EC50 (the concentration giving the half maximal response) for histamine. Thus although histamine binding was potentiated by thiomersal, there was no potentiation of an H_1 receptor-mediated functional response.

Introduction

The binding of agonists to the histamine H_1 receptor is particularly sensitive to reduction of a dithiol bond by dithiothreitol (DTT) (Donaldson & Hill 1986a, 1987) or to covalent modification of a receptor thiol by N-ethylmaleimide (NEM) (Yeramian et al 1985), whereas the binding of antagonists is relatively little altered by either agent (Yeramian et al 1985; Donaldson & Hill 1986a; Hughes et al 2000). The effect of both DTT and NEM is to potentiate H_1-agonist binding, as indicated by a shift to lower concentrations of the curve for agonist inhibition of the binding of [^{3}H]mepyramine. In the case of DTT, the potentiation of the binding of H_1-agonists can be correlated with an increased potency in functional responses (Fleisch et al 1973; Donaldson & Hill 1986b, 1987; Young & Young 1994). No comparable effect has been demonstrated with NEM (Fleisch et al 1973), probably as a consequence of additional actions of NEM at sites post-agonist binding, such as the well established inactivation of G proteins (see Sidhu et al 1991), leading to an overall inhibition of functional responses.

The extent to which the action of NEM is general to agents which modify thiols is not clear. In contrast to NEM, the Group IIB metal cations, Hg^{2+}, Cd^{2+}, and Zn^{2+}, which bind strongly to thiols, are potent inhibitors of antagonist binding to the H_1 receptor, particularly Hg^{2+} (Treherne et al 1991). However, it is not known whether organic mercurials, such as thiomersal (thimerosal), behave in the same way as mercuric cations or as an organic thiol reagent.

There is also a methodological reason why the action of thiomersal on the H_1 receptor is of interest. In some studies on the potentiation by thiomersal of 1,4,5-IP_3-induced Ca^{2+} release from intracellular stores histamine has been used as the agonist to generate 1,4,5-IP_3 via the activation of H_1 receptors (Bootman et al 1992; Young et al 1998; Montero et al 2001). It is clear that there is an effect of thiomersal at the 1,4,5-IP_3 receptor, but the argument has been made that there is no contribution from an action at the H_1 receptor, since 100 μM thiomersal produced a partial

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inhibition of histamine-stimulated accumulation of total [^{3}H]inositol phosphates ([^{3}H]IP) in Li^+-treated cells (Bootman et al 1992). However, the potentiating effect of thiomersal on histamine-induced Ca^{2+} mobilization in U373 MG astrocytoma cells is apparent at a concentration an order of magnitude lower (Young et al 1998) and it is not established whether at this concentration, 10 μM, part of the potentiating effect might not be at the level of the H_1 receptor. We show here that concentrations of thiomersal in the range 3–30 μM do enhance the binding of histamine to the H_1 receptor, but the effect on histamine-stimulated [^{3}H]IP accumulation is still partial inhibition.

Materials and Methods

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

Guinea-pig cerebellar membranes were prepared in Tris-HCl buffer as described previously (Gibson et al 1994). Incubations in 10 mM Tris-HCl buffer, pH 7.5, contained 0.5–0.75 nM [^{3}H]mepyramine, histamine (in a few experiments unlabelled mepyramine), thiomersal (where appropriate) and cerebellar homogenate (approx. 0.2 mg protein) in a total volume of 1 mL (four replicates at each histamine concentration; eight to twelve replicates in the absence of inhibitor, spread through the experiment). Curves in the presence and absence of thiomersal were always determined in parallel in the same experiment. Equilibration was for 60 min at 30 °C and was terminated by filtration through Whatman GF/B glass fibre paper, pre-soaked in 0.3% (w/v) polyethylenimine for 3–5 h, using a Brandel (Gaithersburg, MD) cell harvester. The filters were washed with ice-cold buffer and then transferred to scintillation insert vials containing 4.0 mL scintillator (Emulsifier-Safe, Packard) and allowed to stand at room temperature for at least 3 h before determination of tritium by liquid scintillation counting.

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

U373 MG cells (National Culture Collection, Porton Down, UK) were cultured in Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F-12 (1:1 v/v; Gibco), containing 10% foetal calf serum and 2 mM glutamine (Gibco) and supplemented with 1% (v/v) anti-mycotic/antibiotic mixture (10 000 U mL^{-1} penicillin, 10 mg mL^{-1} streptomycin and 25 μg mL^{-1} amphotericin B; Sigma). Cells were dissociated with trypsin/EDTA (Sigma), seeded (approx. 50 000 cells/well) 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% dialysed 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 off and the cells were equilibrated for 10 min at 37 °C in 1.0 mL HEPES medium (in mM: NaCl 120, KCl 5.4, $MgCl_2$ 1.6, $CaCl_2$ 1.8, HEPES 25 and D-glucose 11, pH 7.4). This was removed, HEPES medium containing LiCl (30 mM) added (0.48 mL) to each well and the cells incubated for 15 min at 37 °C before addition of 20 μL histamine or histamine + 10 μM thiomersal (four replicates at each histamine concentration). Cells were then incubated for a further 30 min before termination of the reaction by aspirating off 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 sample was taken from each well and added to 400 μL of a 1:1 (v:v) solution of trioctylamine/1,1,2-trichlorotrifluoroethane. A portion of the upper phase (0.35 mL) was transferred to an insert vial, 3 mL 50 mM HEPES buffer, pH 7.6, added and the mixture applied to an AG1 X-8 (formate form, 100–200 mesh; Biorad) anion-exchange column. [^{3}H]Inositol and [^{3}H]glycerophosphoinositol were eluted with 10 mL H_2O and 10 mL 60 mM ammonium formate/5 mM sodium tetraborate, respectively, and [^{3}H]inositol mono-, bis- and tris-phosphates ([^{3}H]IP) eluted with 10 mL 0.8 M ammonium formate/0.1 M formic acid. Quicksafe A (10 mL, Zinnser Analytic) was added to the eluant and the tritium content determined by liquid scintillation counting.

Analysis of data

Data for the inhibition of [^{3}H]mepyramine binding by histamine were fitted by non-linear regression to a Hill equation (logistic equation):

$$\%\ \text{of uninhibited binding of [}^3\text{H]mepyramine} = (100 - NS)/((A/IC50)^{nH} + 1) + NS$$

where NS is the percentage of the binding of [^{3}H]mepyramine insensitive to inhibition by histamine, A is the concentration of histamine, nH is the Hill coefficient and IC50 the concentration of histamine giving 50% inhibition of the histamine-sensitive [^{3}H]mepyramine binding.

Concentration–response data for histamine-induced [^{3}H]IP accumulation were similarly fitted to a hyperbola:

$$\text{Response} = \text{Resp}_{max}\ C/(C + EC50)$$

where Resp_{max} is the maximum response, C is the concentration of histamine and EC50 is the concentration giving the half maximal response.

Statistical comparison of the IC50 values of curves of histamine inhibition of [^{3}H]mepyramine binding in the presence and absence of thiomersal 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 (excess sum of squares method (Rodbard 1974)), as described by Young & Young (1994).

The errors of ratios (e.g. % of uninhibited binding) 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.m., was expressed as the weighted mean ± s.e.m. (Colquhoun 1971).

Chemicals

[^{3}H]Mepyramine (30 Ci mmol^{-1}) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) and [^{3}H]inositol (21 Ci mmol^{-1}) from New England Nuclear (Hounslow, Middlesex, UK). Histamine dihydrochloride, HEPES, mepyramine maleate, perchloric acid, phytic acid, thiomersal, 1,1,2-trichlorotrifluoroethane (freon), tri-n-octylamine and Tris were purchased from Sigma (Poole, Dorset, UK).

Results and Discussion

Comparison of the effect of thiomersal, NEM and $HgCl_2$ on [^{3}H]mepyramine binding

Thiomersal produced only a weak inhibition of the binding of [^{3}H]mepyramine (11 ± 1% inhibition at 10 μM and 32 ± 3% inhibition at 300 μM), similar to that produced by NEM (Figure 1). In contrast [^{3}H]mepyramine binding was potently inhibited by $HgCl_2$ (Figure 1), log(IC50) -5.37 ± 0.02 (IC50 4.3 ± 0.1 μM, approximate s.e.), consistent with our earlier observations using the same experimental protocol for Hg^{2+}, IC50 5 μM (Treherne et al 1991), and NEM (Hughes et al 2000). The very steep curve for $HgCl_2$ (best-fit Hill slope 2.69 ± 0.51) was a consequence of an effectively irreversible action and depletion of the free concentration of Hg^{2+} due to extensive tissue binding (R. Dempster & J. M. Young, unpublished observations).

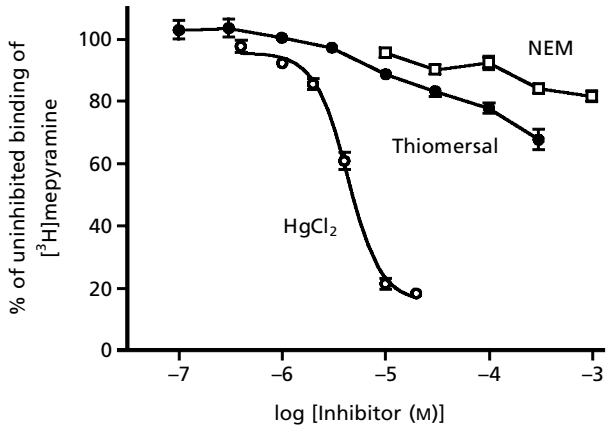

Figure 1 Inhibition of the binding of [^{3}H]mepyramine to guinea-pig cerebellar membranes by thiomersal, $HgCl_2$ and NEM. Points for thiomersal are the weighted means ± approximate s.e.m. from two to nine independent measurements. The data for $HgCl_2$ and NEM are from quadruplicate determinations within a single experiment, each of which was in close agreement with previous observations (Trehene et al 1991; Hughes et al 2000). Where no error bars are apparent the error was within the size of the symbol. The curve drawn for $HgCl_2$ is the best-fit line to a Hill equation (see Materials and Methods).

Effect of thiomersal on histamine binding to the H_1 receptor

The effect of thiomersal, 3–30 μM, on the inhibition of the binding of [^{3}H]mepyramine by histamine was to produce a leftward shift of the inhibition curve to lower concentrations, without any consistent effect on the Hill slope or the extent of the binding of [^{3}H]mepyramine insensitive to inhibition by histamine (illustrated for 30 μM thiomersal in Figure 2A). Neither 3 nor 10 μM thiomersal caused any leftward shift of curves of the inhibition of the binding of [^{3}H]mepyramine by non-radiolabelled mepyramine (two experiments at each concentration; data not shown), but in one of two experiments with 30 μM thiomersal there was a small, 1.3-fold, but statistically significant, shift of the inhibition curve for mepyramine to the right, indicating a reduction in the affinity of mepyramine. This effect was more pronounced with 100 μM thiomersal, and therefore measurements of the effect of thiomersal on the histamine curve were not made at concentrations greater than 30 μM. The extent of the shift of the IC50 for histamine as a function of the concentration of thiomersal is shown in Figure 2B.

The extent of the shift of the histamine inhibition curves by 10–30 μM thiomersal was similar to that produced by 1–2 mM NEM (Yeramian et al 1985; our own unpublished observations). In the original experiments with NEM (Yeramian et al 1985), as with those with DTT (Donaldson & Hill 1986a), the shift was accompanied by a decrease in the Hill coefficient, which was interpreted as an increase in the proportion of a high affinity state of the receptor. This high affinity state has been presumed to represent the ternary complex between agonist, receptor and G protein and an increase in the amount of this complex would seem likely to be associated with an enhanced functional response. However, it has been questioned whether Hill slopes < 1 for agonist inhibition curves are adequately accounted for by the ternary complex model, particularly with regard to the effects of modulators such as NEM (Lee et al 1986), and the observation that there was no significant effect on the slope of the histamine inhibition curves in the presence of 3–30 μM thiomersal (mean values of the Hill coefficient: 0.77 ± 0.02 in control curves and 0.79 ± 0.02 in the presence of 1–30 μM thiomersal, six experiments) should be interpreted with caution with regard to receptor function.

Effect of thiomersal on histamine-stimulated inositol phosphate accumulation

To investigate directly the effect of 10 μM thiomersal on H_1-receptor activation of phospholipase C, we measured histamine-induced inositol phosphate accumulation in human U373 MG astrocytoma cells in the presence and

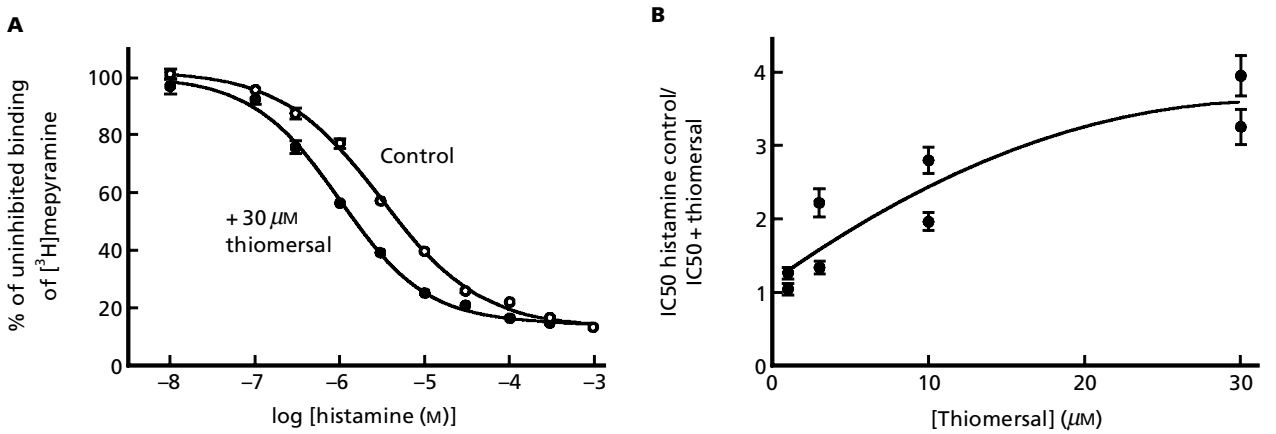

Figure 2 Enhancement by thiomersal of the binding of histamine to the histamine H_1 receptor. A. Inhibition by histamine of the binding of 0.61 nM [^{3}H]mepyramine in the presence or absence of 30 μM thiomersal. Points are the means ± approximate s.e.m. from quadruplicate determinations within a single experiment. Where no error bars are apparent the error was within the size of the symbol. The curves drawn are the best-fit lines to a Hill equation (see Materials and Methods). Best-fit parameters ± estimated s.e. in the absence and presence of thiomersal: Hill coefficient 0.77 ± 0.03 and 0.77 ± 0.03, IC50 3.37 ± 0.16 and 1.04 ± 0.06 μM, non-specific binding 13.1 ± 0.6 and 13.5 ± 0.5%, respectively. B. Concentration dependence of the extent of the enhance of histamine binding by thiomersal. Points are the ratios of the best-fit IC50 values for histamine inhibition of [^{3}H]mepyramine binding in the absence and presence of thiomersal ± approximate s.e. taken from independent experiments (such as that shown in A above). All shifts were statistically significant, as assessed by the excess sum of squares test (see Analysis of data), except in one experiment with 1 μM thiomersal. The curve drawn is a best-fit polynomial and is solely to emphasize the trend of the data.

absence of thiomersal. Previously, we have shown that in this cell line [^{3}H]IP formation in response to histamine concentrations of 1 mM and below was mediated by the H_1 receptor (Arias-Montaño et al 1994) and that there was no evidence for the presence of functional H_2 receptors, which might modulate this response (Wong et al 2000).

Histamine alone produced an approximately 20-fold increase in [^{3}H]IP over a 30-min incubation (Figure 3). This was reduced in the presence of 10 μM thiomersal (best-fit values of $Response_{max}$: 20.6- ± 1.2-fold and 12.9- ± 1.4-fold stimulation in the absence and presence of thiomersal, respectively), without any significant effect on log(EC50) for histamine, −5.49 ± 0.12 and −5.57 ± 0.24, respectively. Thiomersal (10 μM) alone had no consistent effect on basal [^{3}H]IP accumulation (570–1040 dpm) over the three experiments.

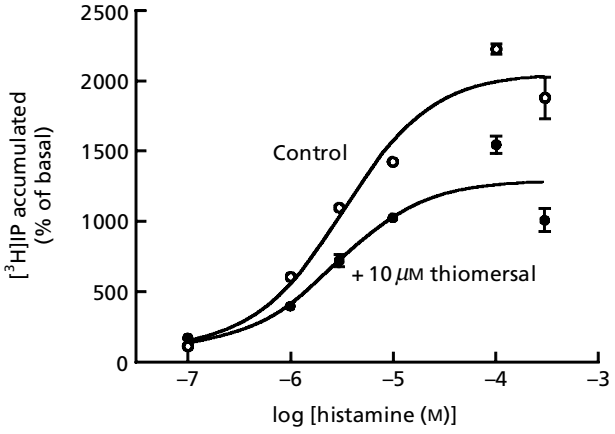

Figure 3 Effect of 10 μM thiomersal on histamine stimulated [^{3}H]IP accumulation in monolayers of U373 MG astrocytoma cells. Values are the weighted means ± s.e. from three independent experiments (one to three determinations at each concentration). Where no error bars are apparent the error was within the size of the symbol. The curve drawn is the best-fit line to a hyperbola (best-fit values of EC50 and B_{max} are given in the text).

Conclusions

Two principle conclusions can be drawn from the data. Firstly, thiomersal, an organic mercurial which will bind to thiol groups, is a weak inhibitor of [^{3}H]mepyramine binding, but enhances the binding of histamine to the H_1 receptor. In this respect thiomersal acts in the same way as NEM and not as the free Hg^{2+} ion. Potentiation of H_1-agonist binding may thus be an effect shared by organic thiol reagents. Secondly, the enhanced binding in the presence of 10 μM thiomersal is not reflected in any increase in histamine-induced [^{3}H]IP accumulation: on the contrary there is an inhibition of the maximum response. This may reflect a second action of thiomersal at the level of G protein coupling, analogous to the action of NEM, or at a post-receptor site.

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