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Analysis of an H_1 receptor-mediated, zinc-potentiated vasoconstrictor action of the histidyl dipeptide carnosine in rabbit saphenous vein

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1 The contractile action of the dipeptide carnosine (β-alanyl-L-histidine), active as a Zn•carnosine complex (Zn•Carn), was investigated in isolated rings of rabbit saphenous vein (RSV) and was found to be antagonized by the H_1 antagonist mepyramine.

2 Mepyramine-sensitive, histamine-induced contractures in RSV, were smaller ($73 \pm 0.1\%$) and less well sustained than carnosine-induced contractures.

3 Schild plot values for mepyramine antagonism were, for carnosine-induced contractures; $pA_2 = 7.97 \pm 0.12$, slope $= 1.33 \pm 0.06$ ($r = 0.793$) and for histamine-induced contractures; $pA_2 = 8.48 \pm 0.07$, slope $= 0.63 \pm 0.05$, $r = 0.957$).

4 Serotonergic antagonists methiothepin and ketanserin, antagonize both carnosine- and histamine-induced contractures in RSV, probably reflecting coincidental inhibition at the H_1-receptor.

5 Carnosine, with Zn present, can inhibit the H_1-specific binding of [^{3}H]-mepyramine to isolated guinea-pig cerebellar membranes (log IC_{50}s -2.78 ± 0.02, -3.93 ± 0.03 and -4.64 ± 0.03 at 10, 30 and 80 μM Zn respectively; values corrected for the Zn-specific inhibition which has a logIC_{50} of -4.20).

6 In the radioligand binding assay, the effect of carnosine can be described as a function of Zn•Carn concentration with an apparent logIC_{50} of -5.61. This value is consistent with that obtained from the functional studies on RSV.

7 Histamine-induced contractures have an indomethacine-sensitive component ($27.2 \pm 8.3\%$ of control response), not apparent with carnosine-induced contractures.

8 Like histamine, carnosine evoked an H_2-mediated (cimetidine-sensitive) relaxation in the presence of mepyramine, but was less potent ($10.8 \pm 3.1\%$ residual tension at 10 mM carnosine compared with $13.4 \pm 7.5\%$ at 0.1 mM histamine).

9 Carnosine, like mepyramine, can 'reveal' the H_2-mediated relaxation of histamine providing further evidence that carnosine binds at the H_1 receptor.

10 We conclude that carnosine can act at the smooth muscle H_1-receptor to provoke vasoconstriction and that it also has the potential to act at H_1-receptors in CNS.

Keywords: Carnosine; histamine; H_1 receptor; H_2 receptor; mepyramine; cimetidine; rabbit saphenous vein; guinea-pig cerebellum; vasoconstrictor

Introduction

The endogenous histidyl dipeptide carnosine (β-alanyl-L-histidine) is found, often at high concentrations, in a range of tissues, particularly skeletal muscle, brain and heart (e.g. Flancbaum *et al*., 1990b). A range of actions of carnosine has been reported over the years (O'Dowd *et al*., 1996), but its physiological role or roles remain unclear. Interest in carnosine as a putative vasoactive agent follows from the observation reported over 60 years ago (Mason & Binkley, 1931; du Vigneaud & Hunt, 1936) that bolus injections of carnosine *in vivo* caused a transient fall in blood pressure. We recently reported that carnosine can induce large, sustained contractures in isolated rings of rabbit saphenous vein (RSV) and that the effect is attributable to the Zinc•carnosine complex (Zn•Carn; EC_{50} 7.4×10^{-8} M, O'Dowd *et al*., 1996). The maximum tension evoked by Zn•Carn is approximately double that for an optimal concentration of noradrenaline. The effect is specific; related dipeptides anserine (β-alanyl-1-methyl L-histidine) and homocarnosine (γ-aminbutyryl L-histidine) are ineffective, as are carnosine's constituent amino acids histidine and β-alanine. Likewise, although a number divalent cations can bind to carnosine, only Zn^{2+} facilitated the vasoconstrictor action of carnosine. The action of carnosine on vascular smooth muscle is thus virtually unique, the agonist being a dipeptide acting in the form of a metal ion complex.

The speed and selectivity of action of carnosine (or apparently Zn•Carn) suggested a receptor-mediated pathway. In preliminary tests, carnosine-induced contractures proved sensitive to a range of 5-HT antagonists, including ketanserin, cinanserin and methiothepin (O'Dowd *et al*., 1996), suggesting that a '5-HT_1-like' receptor (Martin & MacLennan, 1990, Van Heuven-Nolsen *et al*., 1990) might be involved. However, to extend the analysis, and recognizing structural similarities between carnosine and histamine, we have now investigated the contribution of the H_1-receptor. We report here the effect of the high affinity histamine H_1 antagonist mepyramine (Ash & Schild, 1966) on carnosine-induced contractures and of carnosine on H_1-specific binding of [^{3}H]-mepyramine to isolated guinea-pig cerebellar membranes. These results have led us to question the selectivity of the previously used 5-HT antagonists with respect to the H_1-receptor; therefore, it

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proved necessary to investigate their effect on histamine-induced contractures.

Histamine-induced contractures are H_1-mediated in RSV (Hill, 1990), while histamine-induced relaxation has been demonstrated in pre-contracted vessels in the presence of an H_1 antagonist (Tsuru *et al*., 1983; Schoeffter & Godfraind, 1989), an action which is attributed to a smooth muscle H_2 receptor which is blocked by cimetidine (Brimblecombe *et al*., 1975). Consistent with this is the observation that cimetidine, at concentrations of 1–10 μM, can potentiate the H_1-mediated contractile response of histamine in some tissues, including isolated human saphenous vein (Schoeffter & Godfraind, 1989). We investigated whether carnosine might also demonstrate an H_2 receptor-mediated relaxation in saphenous vein during H_1 blockade and examined the effect of cimetidine (10 μM) on the carnosine-induced contractile response.

It has been reported that the cyclooxygenase inhibitor, indomethacin, has an inhibitory effect on histamine-induced contraction in some smooth muscle preparations (Schoeffter & Godfraind, 1989), suggesting the participation of constricting prostanoids. The possibility of an analogous effect in the case of the carnosine-induced contractile response was, therefore, examined.

Preliminary results have been presented to the Physiological Society (O'Dowd & Miller, 1996).

Methods

Organ bath experiments

Vascular rings (2–3 mm long; either freshly dissected or dissected and kept overnight at 4°C) from the lateral saphenous vein of the rabbit (New Zealand White, 2.2–2.4 kg.wt., sacrificed by i.v. overdose of sodium pentobarbitone (Euthatal), 120 mg kg^{-1}) were mounted for isometric tension recording in standard organ baths (15 ml) containing normal physiological salines (in mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, D-Glucose 11.1, at 37°C, equilibrated with 95% O_2 and 5% CO_2 (for details see Daly *et al*., 1988). Tension signals were recorded and analysed using MacLab 8 (A.-D. Instruments) hardware and software.

Protocol Preparations were mounted under an original resting tension of 1.5 g.wt. and allowed to relax. Final resting tension was 0.3–0.5 g.wt. After an equilibration of 1 h, the bathing solutions were changed once and a further 1 h equilibration period was allowed before commencing the experimental regime. All tissues (except those reported in Figure 3) were pre-exposed to a single submaximal dose of histamine (30 μM) or carnosine (0.1 mM in the presence of 5 μM Zn) before the main protocol was started. Zn was added as a solution of the acetate salt, at 0.1% of bath volume.

Antagonist experiments

Preparations were cumulatively activated with carnosine (1 μM–10 mM) or histamine (1 μM–1 mM). After washout, preparations were incubated for 30 min with mepyramine (1–100 nM), cimetidine (10 μM) or indomethacin (5 μM) and the cumulative exposure repeated; each preparation thus acted as its own control. One preparation in each set had no antagonist added to control for changes in tissue sensitivity with time. Zinc (5 μM) was always included in protocols involving carnosine to ensure an initial higher sensitivity to the agonist (O'Dowd *et al*., 1996). To ensure comparability between results, 5 μM Zn was also included in the antagonist protocol for histamine and mepyramine.

The effects of reputed serotonergic antagonists methiothepin (0.5–5 nM) and ketanserin (10–100 nM) on histamine contractures were also investigated (these were previously found to inhibit carnosine contractures). In both cases cimetidine (1 μM) was included in the bathing solutions to minimize H_2-mediated effects. In the case of methiothepin a slightly different protocol was used whereby a single concentration-response curve (CRC) was constructed 30 min after addition of antagonist; separate preparations to which no antagonist was added served as the controls.

Relaxation experiments

Preparations were pre-contracted with noradrenaline (NA) in the presence or absence of mepyramine (10 μM) which was added 20 min prior to NA exposure. When tension had stabilized, histamine or carnosine was applied cumulatively, followed by cimetidine (10 μM). In a different protocol, carnosine (1–10 mM) was used as the contractile agent (no mepyramine present) prior to the addition of histamine.

Radioligand binding studies

These experiments were carried out essentially as described by Gibson *et al*. (1994). Dunkin-Hartley guinea-pigs (male, 300–400 g) were killed by a blow to the head and the cerebella removed. Tissue was homogenized for a few seconds, using a Polytron homogenizer, in 20 vol. ice cold 10 mM HEPES buffer, pH 7.5. The homogenate was centrifuged at 17,000 × g and 4°C for 30 min, the pellet re-suspended in buffer, and the centrifugation repeated to produce a final pellet which was re-suspended in buffer (7 mg wet wt/ml) and either used immediately or frozen at −20°C for future use.

To determine H_1-specific binding, [^{3}H]-mepyramine (final concentration 0.4–0.7 nM), unlabelled compounds, membrane suspension and Zn^{2+} ions were added to assay tubes in triplicate (final incubation volume 1 ml). Assay buffer throughout was 10 mM HEPES, pH 7.5. Non-specific binding was determined as that insensitive to 2 μM triprolidine (Chang & Snyder, 1980). The tubes were vortex mixed and incubated at 30°C for 60 min whereafter the contents were filtered immediately through Whatman GF/C filter paper, pre-soaked in 0.3% (w/v) polyethylenimine (Sigma U.K.) for 30 min, using a Brandel cell harvester. The filters were subjected to three 4 ml washes with ice-cold assay buffer then transferred to scintillation tubes containing 4 ml of scintillation fluid (Ecoscint A, National Diagnostics). Samples stood overnight and were then counted for tritium in a liquid scintillation counter. Specific binding was defined as the radioactivity bound after subtraction of non-specific binding as defined by 2 μM triprolidine.

Drugs

Carnosine (free base), histamine dihydrochloride, triprolidine and noradrenaline bitartrate were purchased from Sigma U.K. (Poole, Dorset). Mepyramine maleate, methiothepin maleate and ketanserin tartrate were from Tochris-Cookson (Bristol, U.K.), cimetidine from SKB (Welwyn Garden City, U.K.) And [^{3}H]-mepyramine (28 $Cimmol^{-1}$) from Amersham International.

In the organ bath experiments, all drugs were added from stock solutions in distilled water, except for indomethacin which was in ethanol, at 0.1% of bath volume. Stock solutions of carnosine and histamine were adjusted to pH7.4. In both organ bath and radioligand studies, Zn was added in the form of zinc acetate.

Data analysis

Data for accumulated and derived parameters are quoted as means ± s.e.mean; n = number of preparations from different animals. Dose-response curves were fitted, for individual preparations, to the Hill equation with the maximum constrained to unity, or derived from the best-fit, as appropriate. In general, values for EC_{50} (or IC_{50}) were calculated as geometric means (arithmetic mean of $logEC_{50}$) of pooled results, but in some cases, where appropriate, a single best-fit curve was constructed using mean pooled data. The significance of any differences between these parameters for individual test and control preparations was assessed by Student's t-test (2-tailed), with significance assumed for $P \leqslant 0.05$. pA_2 values were calculated using Schild analysis (Arunlakshana & Schild, 1959).

The concentration of the Zinc•Carn complex was calculated using a multi-ligand, multi-ion program ('REACT' by Prof G.L. Smith, University of Glasgow) according to principles described elsewhere (Smith & Miller, 1985) developed for work with chemically 'skinned' muscle fibres. The relevant stoichiometric affinity constants were employed (Sillén *et al*., 1974; Pettit & Powell, 1993).

Results

Effect of mepyramine on contractile responses of carnosine and histamine

Mepyramine (10 and 100 nM, $n=6$) produced a concentration-dependent rightward shift in the carnosine CRC ($P \leqslant 0.03$) with no associated alteration in the maximum response (Figure 1A); 1 nM produced no significant shift. Histamine (10 μM – 1 mM, with 5 μM Zn present, as for carnosine) produced similar contractures which were also antagonized by mepyramine (1 – 100 nM, significant for 10 and 100 nM, $P \leqslant 0.0006$; Figure 1B, $n=4$ or 5). Histamine-induced contractures are transient (not shown), whereas carnosine-induced contractures are well-sustained. Results in Figures 1A and B are expressed as the means ± s.e.mean of the second set of responses normalized to their own control maximum response. Values (means ± s.e.mean) for $logEC_{50}$, were; -4.00 ± 0.14 (carnosine alone, $n=6$), -3.59 ± 0.05 (10 nM mepyramine, $n=6$) and -2.69 ± 0.07 (100 nM mepyramine, $n=7$). For histamine, the values were; -4.42 ± 0.02 (histamine alone, $n=6$), -4.30 ± 0.08 (1 nM mepyramine, $n=4$), -3.90 ± 0.04 (10 nM mepyramine, $n=5$) and -3.50 ± 0.05 (100 nM mepyramine, $n=4$). (Control results were scaled to 100% in the case of histamine).

Values determined from Schild plot analysis (Figure 2A and B) were, for carnosine; $pA_2 = 7.97 \pm 0.12$, slope $= 1.33 \pm 0.06$ ($r = 0.793$) and for histamine; $pA_2 = 8.48 \pm 0.07$, slope $= 0.63 \pm 0.05$, ($r = 0.957$). These pA_2 values are significantly different from each other ($P < 0.005$). Both slopes are significantly different from unity ($P < 0.004$) indicating that the antagonism is not of a simple competitive nature in either case (see Discussion).

Effect of cimetidine and indomethacin

The maximal contractile response of histamine was depressed (by $27.2 \pm 8.3\%$ of control, $n=4$) in the presence of indomethacin (5 μM) (data not shown). By contrast, the carnosine response ($107 \pm 5.3\%$ of initial control) was unaffected by indomethacin ($110 \pm 4.5\%$; $n=6$). Cimetidine (10 μM) had no effect on the carnosine contractile response ($n=6$), but shifted the histamine CRC leftwards ($logEC_{50}$s -4.95 in the presence of cimetidine *vs* -3.85 for

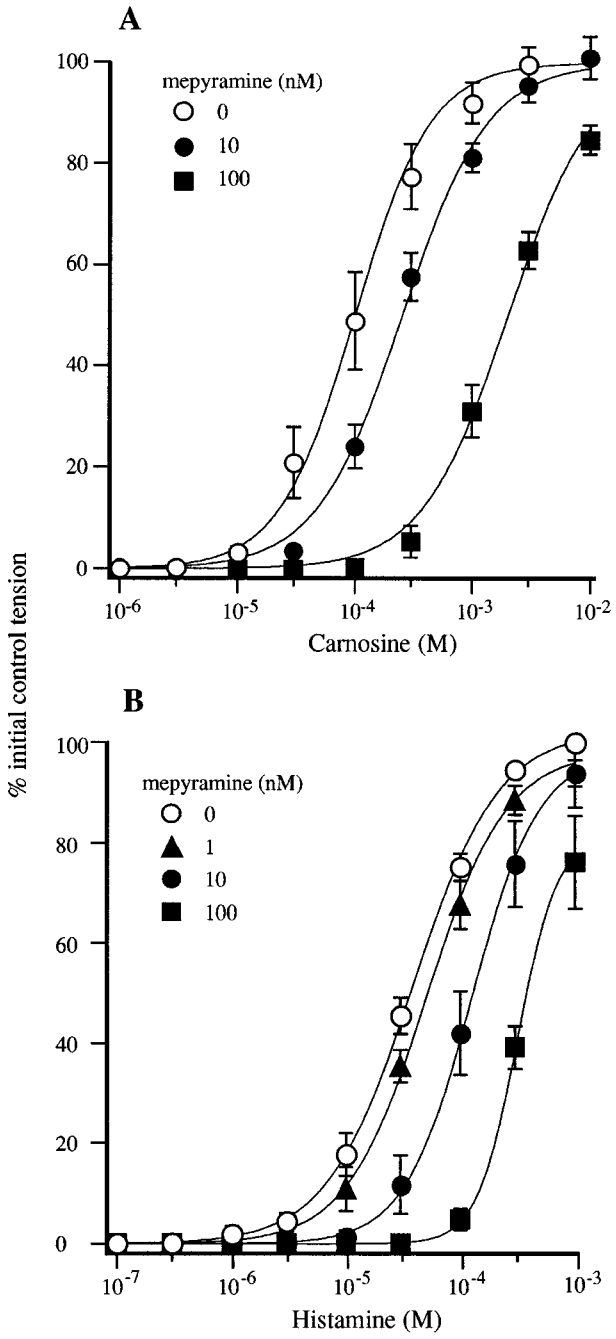

Figure 1 Antagonism of carnosine-induced (A) and histamine-induced (B) contractile responses by mepyramine. Steady-state tension (means ± s.e.mean expressed as a percentage of the initial controls; see Methods) plotted against carnosine concentration (A) and histamine concentration (B); 5 μM Zn present in both throughout. Values (means ± s.e.mean) for $logEC_{50}$ and number of preparations are shown in the text. See text also for statistical analysis.

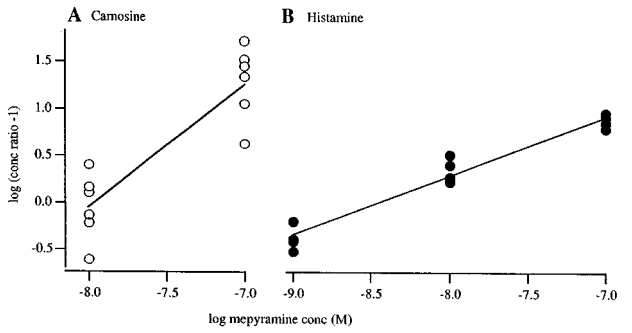

Figure 2 Schild plots of mepyramine antagonism of carnosine and histamine. Linear regression best-fit lines are shown against collected data for carnosine (A; $n=6$) and histamine (B; $n=4$). See text for regression values.

control, $n=2$; data not shown) as previously described by others (Shoeffter & Godfraind, 1989).

Effect of methiothepin and ketanserin histamine-induced contractures

Histamine-induced contractures proved to be sensitive to inhibition by ketanserin and especially by methiothepin (Figure 3A and B; cimetidine 1 μM included to block H_2-receptor-mediated relaxation). Although the histamine CRC is shifted to the right by both antagonists, a depression of the maximum response contributes to the contractures being largely blocked by 10–100 nM ketanserin (Figure 3A) and almost completely blocked with as little as 5 nM methiothepin (Figure 3B). The large variability in sensitivity to ketanserin, evident from the error bars in Figure 3A, reflects that in some preparations blockage was complete at all the histamine concentrations tested, but in others a significant contracture persisted. Values for $\log EC_{50}$ and maximum response (as percentage of control maximum) respectively were, for ketanserin; −4.62 and 101.1 (histamine alone), −4.21 and 27.3 (10 nM) and −3.68 and 36.0 (100 nM). The corresponding values for methiothepin were; −4.88, 101.3 (histamine alone), −4.89, 73.8 (0.5 nM), −4.63, 45.7 (1 nM) and −2.95, 18.5 (5 nM).

Radioligand binding studies

Non-specific binding under the assay conditions employed was typically 3–5% of total binding. The ability of carnosine, (alone and in the presence of Zn), histamine and Zn alone to inhibit H_1-specific binding of [^{3}H]-mepyramine binding to cerebellar membranes was determined. In agreement with a previous report (Treherne *et al*., 1991) Zn itself was found to inhibit binding of [^{3}H]-mepyramine ($\log IC_{50}$ −4.20; Figure 4). The combined effect of Zn and carnosine is shown in Figure 5. In Figure 5A the total percentage displacement by these agents is shown with respect to the total carnosine concentration. In order to correct for the displacement attributable to Zn (as shown in Figure 4) and to reveal carnosine-specific inhibition of [^{3}H]-mepyramine (Figure 5B), results were adjusted (using values for Zn inhibition obtained in the same experiment). In the absence of Zn, carnosine was virtually ineffective ($\log IC_{50}$ −1.48, Figure 5B). However, in the presence of Zn (10, 30, 80 μM) carnosine-specific inhibition of [^{3}H]-mepyramine binding was observed (Figure 5B; see Table 1 for values). By analogy to our treatment of results from the blood vessel studies (O'Dowd *et al*., 1996), we were able to re-express

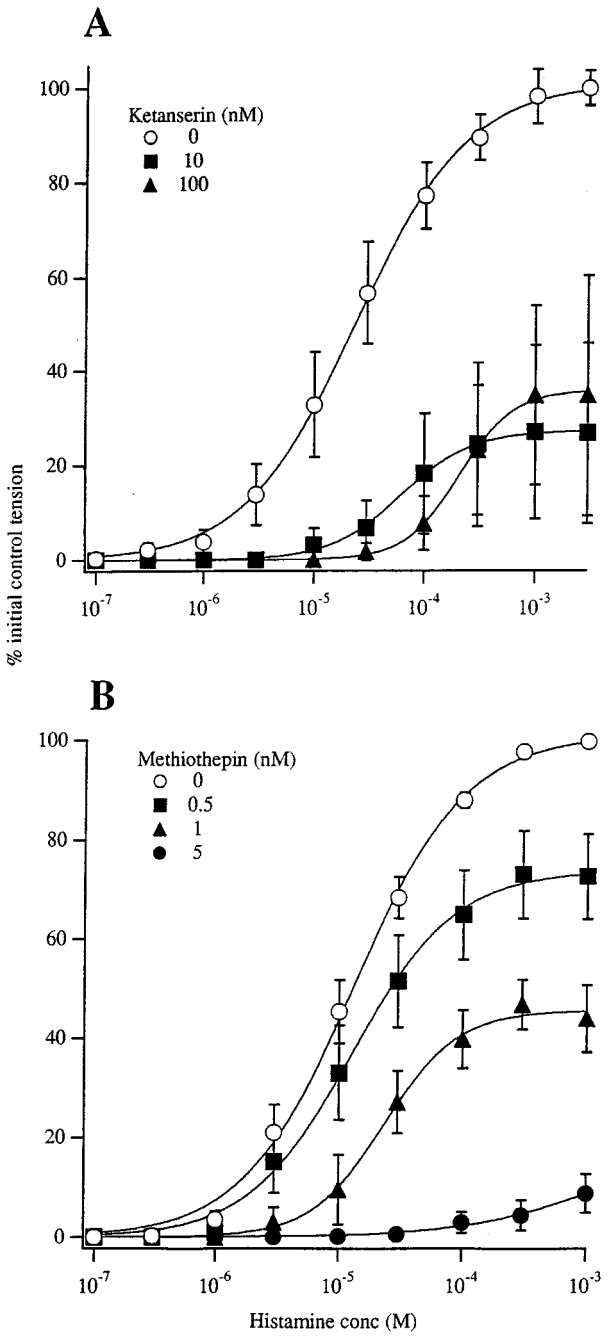

Figure 3 Antagonism of histamine-induced contractile response by ketanserin (A) and methiothepin (B). Steady-state tension (means ±s.e.mean) plotted against histamine concentration (1 μM cimetidine present in both throughout). In the case of ketanserin, data were expressed as a percentage of initial control maximum in the same preparation (re-scaled to 100% since desensitization occurs between first and second exposures to histamine); in the case of methiothepin, separate preparations to which no antagonist was added served as controls. For ketanserin, $n=5-8$ preparations from five animals. For methiothepin, $n=6-10$ preparations from four animals. Values for $\log EC_{50}$ and maximum response are shown in the text.

these data in terms of the concentration of the Zn-carnosine complex using the relevant affinity constants under assay conditions (Figure 5C). When expressed in this way, the Zn 'correction factor' applied necessarily becomes more complicated because binding of Zn to carnosine decreases the concentration of free Zn as the concentration of carnosine is increased. The $\log IC_{50}$ for Zn•Carn was −5.61 with Hill coefficient 0.87 for the best fit curve in Figure 5C. The $\log IC_{50}$

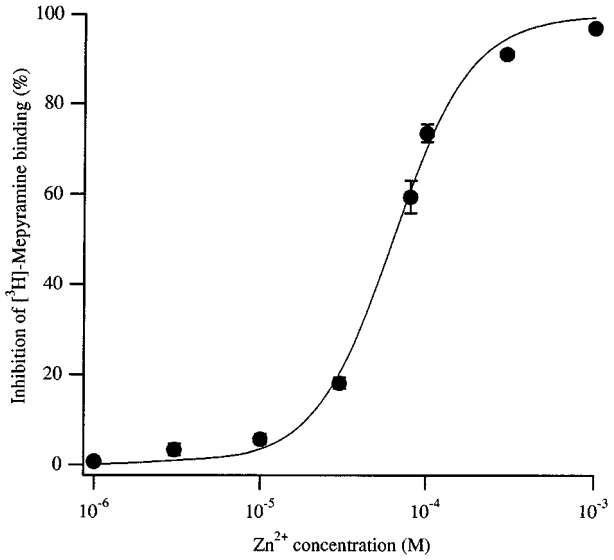

Figure 4 Effect of Zn^{2+} binding of [^{3}H]-mepyramine to the H_1-receptor in isolated Guinea-pig cerebellar membranes. Zn^{2+} inhibited [^{3}H]-mepyramine binding with logIC_{50} −4.20, Hill coefficient 1.86. A single best fit curve was drawn through the data ($n=4-12$ for each Zn concentration) using the Hill equation. Within individual experiments, each determination was carried out in triplicate. Where no error bars are shown the error was smaller than the symbol.

obtained for histamine alone was −5.54 with Hill coefficient 0.78 (data not shown).

Relaxation responses induced by carnosine and histamine

The relaxation response of histamine on pre-contracted blood vessels has been described by others. We have confirmed this effect in saphenous vein preparation using NA as the contractile agent in the presence of the H_1 antagonist mepyramine (10 μM; example trace Figure 6B). Figure 6C shows collected data; at 10 μM and 100 μM histamine the amount of initial tension remaining (means ± s.e.mean) was 40.1 ± 14.9% ($n=5$, $P=0.01$) and 13.4 ± 7.5% (maximal effect, $n=8$, $P<0.0001$) respectively. In the absence of mepyramine (Figure 6A), histamine produced a further increase in tension (101.5 ± 8.1% at 0.1 mM, $n=8$ and 115.6 ± 11.2% at 1 mM, $n=6$, not significant).

Carnosine was also able to elicit relaxation in the presence of mepyramine (example trace Figure 7B) but was less potent (73.1 ± 11.4% of initial tension at 1 mM, $n=6$, $P<0.05$ and 10.8 ± 3.1% at 10 mM, $n=5$, $P<0.0001$; Figure 7C). In the absence of mepyramine (Figure 7A), carnosine produced a substantial further increase in tension (155 ± 10.2% at 1 mM, $n=7$, $P<0.002$ and 179 ± 11.6% at 10 mM, $n=7$, $P<0.0005$).

The relaxing actions of both histamine and carnosine could be partially reversed by cimetidine at 10 μM, (tension restored to 40.1 ± 7.0% of original for histamine, $n=5$, $P<0.01$ and 51.1 ± 7.1% for carnosine, $n=4$, $P<0.005$, see Figures 6C and 7C). Addition of Zn ions (5–30 μM) did not appear to enhance the relaxing action of either agonist (data not shown).

The H_2-mediated relaxing effect is normally only 'revealed' in the presence of an H_1 antagonist such as mepyramine. We were able to show that carnosine, at maximal or near maximal contractile concentrations (1–10 mM), is also able to reveal the relaxing action of histamine (example trace Figure 8A). At 1 μM and 10 μM histamine, the amount of original carnosine-induced tension remaining (means ± s.e.mean) was 64.0 ± 7.0%, $n=10$ ($P<0.0003$) and 17.2% ± 4.1%, $n=12$ ($P<<0.0001$) respectively (Figure 8B). This relaxation could also be reversed by cimetidine (to 57.4 ± 10.0% of original, $n=6$, $P<0.006$ at 10 μM and to 70.4 ± 8.3%, $n=4$, $P<0.003$ at 0.1 mM; Figure 8A and B).

Discussion

We have previously reported (O'Dowd *et al*., 1996) that the endogenous histidyl dipeptide carnosine, in the form of a Zn•Carn complex, can potently contract vascular smooth muscle *via* a receptor-mediated mechanism. Initial observations revealed that the response was unaffected by α-adrenergic antagonists rauwolscine and prazosin, was moderately sensitive to the 5-HT_2 antagonist ketanserin ($pA_2=7.2$) and very sensitive to the 5-HT_1-like antagonist methiothepin ($pA_2=9.4$; both pA_2 values derived from data published in Figure 6, O'Dowd & Miller, 1996). This profile suggested an atypical 5-HT_1-like receptor, which mediates 5-HT-induced contraction in RSV (Martin & MacLennan, 1990; Van Heuven-Nolsen *et al*., 1990). However, we report here that the H_1 antagonist mepyramine can also inhibit carnosine-induced contractures. Unlike the serotonergic antagonists which are individually relatively non-selective, mepyramine is very selective for the H_1 receptor at concentrations less than 100 nM (Hill, 1990). Furthermore, histamine (decarboxylated L-histidine) and carnosine (*β*-alanyl-L-histidine) have some structural similarity, since both contain an imidazole ring. Histamine itself was found to elicit mepyramine-sensitive contractures in RSV although these were smaller and less-well sustained than carnosine-induced contractures; this may be due to the more potent H_2-mediated relaxing effects of histamine which are discussed below. In contrast, 5-HT does not easily elicit a response in the isolated saphenous vein under the same conditions (O'Dowd *et al*., 1996).

Schild analysis of mepyramine antagonism of the histamine and carnosine contractile responses yielded pA_2 values of 8.47 and 7.97 respectively; reported pA_2 values for mepyramine in venous preparations are generally between 8 and 9 (Schoeffter & Godfraind, 1989; Tsuru *et al*., 1983). (The present pA_2 and slope values for histamine/mepyramine antagonism are, in fact, identical to those obtained by Schoeffter & Godfraind in human saphenous vein). In both cases the slopes obtained are significantly different from unity indicating non-competitive antagonism. A slope of <1 can be the result of several factors, including receptor heterogeneity and secondary release of an endogenous agonist (Kenakin, 1984). In the case of histamine this could be due to action of a contractile prostanoid (or other agent). For carnosine (slope >1), there was no evidence of an indomethacin-sensitive component in the contractile response. In addition, the true agonist in this case is Zn•Carn, the concentration of which does not rise in simple proportion to that of carnosine, thus tending to increase the slope of the Schild plot. For both carnosine and histamine any H_2-effect will tend to increase the slope. The unpredictable net effect of these various factors upon the Schild analysis means that the results are not inconsistent with the binding of histamine and carnosine at the same (i.e. H_1) receptor.

We have additionally been able to confirm our prediction that histamine-induced contractures, like carnosine-induced contractures, would prove sensitive to both ketanserin and methiothepin. The results in Figure 3 show that the sensitivity of histamine-induced contractures to these antagonists is, if anything, greater than that of carnosine-induced contractures (O'Dowd *et al*., 1996; Figure 6). These findings suggest that the sensitivity of carnosine-induced contractures to these 'seroto-

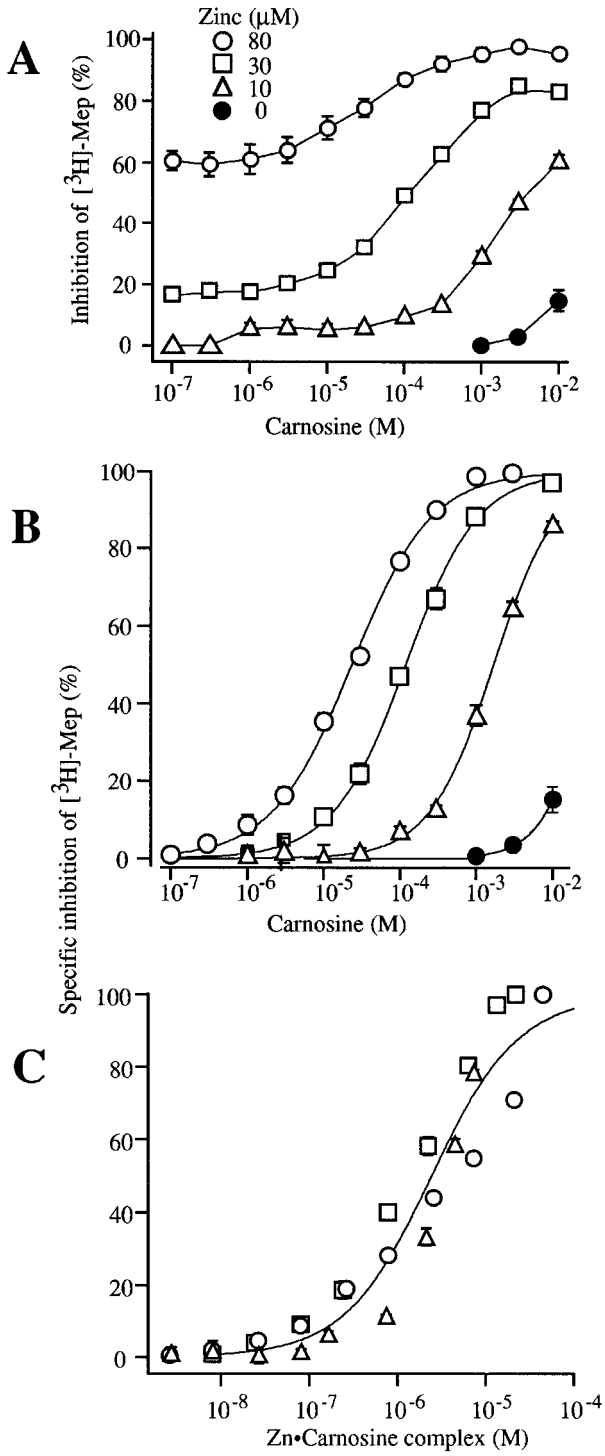

Figure 5 Carnosine-induced inhibition of [^{3}H]-mepyramine binding to the H_1-receptor in isolated guinea-pig cerebellar membranes. (A) Effect of carnosine on H_1 binding in the absence and presence of Zn. The combined effect of carnosine and Zn itself (as percentage inhibition, means ± s.e.mean) at three different concentrations of Zn, plus Zn-free carnosine is shown. (B) Carnosine-specific effect on H_1 binding in the absence and presence of Zn. Carnosine-specific inhibition was calculated by subtracting the percentage inhibition due to Zn alone (see Figure 4) and re-normalizing the results. In the absence of Zn, carnosine is virtually ineffective (see Table 1 for values); a single best-fit curve was drawn through the data for 9–12 determinations using the Hill equation. For results in the presence of Zn, IC_{50}s were calculated as the geometric mean (arithmetic mean of logIC_{50}) of pooled results (all fitted to the Hill equation) from three or more experiments as indicated (see Table 1). Within individual experiments, each determination was carried out in triplicate. Where no error bars are shown the error was smaller than the symbol. (C) Radioligand binding data from B re-plotted as a function of the concentration of Zinc•carnosine complex. This involved revising the %-inhibition values used for A and B to take full account of the reduction in free Zn caused by carnosine binding and thus the necessary re-adjustment for inhibition by Zn ions (from Figure 4). Values for concentration of the Zinc•carnosine complex were calculated as described in Methods. Results obtained at the three added Zn concentrations (10, 30 and 80 μM) can thus be described by a single curve (logIC_{50} −5.61, Hill coefficient 0.87). Total number of

nergic' antagonists was coincidental. The promiscuity of some serotonergic antagonists has previously been reported; an azide derivative of ketanserin can irreversibly bind to the H_1

Table 1 Effect of Zn on inhibition of [^{3}H]-mepyramine binding by carnosine

$[Zn^{2+}]$ (μM)	*log IC_{50}*	n_H	*No. of experiments*
0	−1.48	1.44	9–12
10	−2.78 ± 0.02	1.03 ± 0.01	3
30	−3.93 ± 0.03	0.91 ± 0.01	4
80	−4.64 ± 0.03	0.83 ± 0.04	5

Results were adjusted to take account of the effect of Zn alone. Values (means ± s.e.mean), for logIC_{50}, Hill coefficient (n_H) and for the number of experiments are shown. Data are illustrated in Figure 5B and re-worked in Figure 5C.

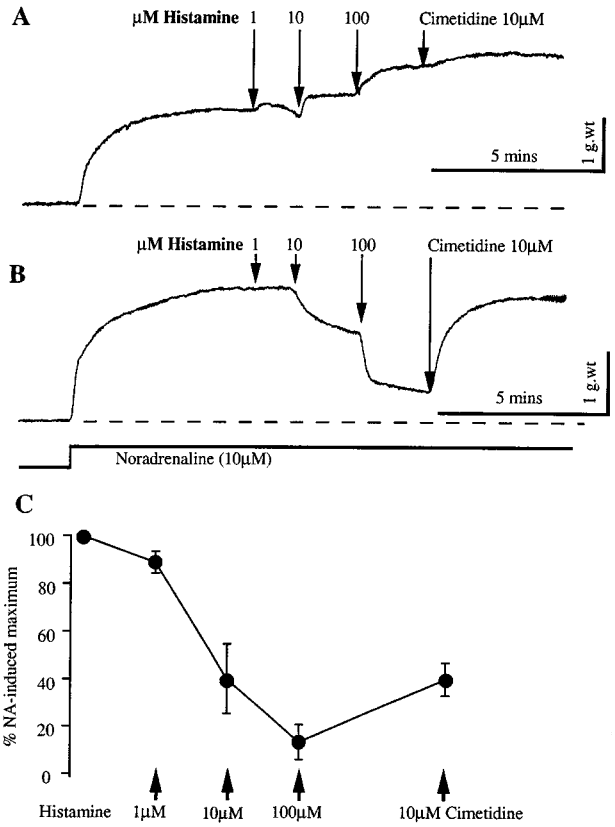

Figure 6 H_2-mediated relaxing effect of histamine. Example traces of the isometric response of two saphenous vein rings to histamine. The tissues were pre-activated by 10 μM NA and tension allowed to stabilize. Three levels of histamine, followed by one of cimetidine were applied (as indicated above the traces), either in the absence (A) or the presence (B) of 10 μM mepyramine. (C) shows collected data (means ± s.e.mean $n = 5$ to 8) for the responses obtained in the presence of mepyramine (i.e. with the protocol in B). See text for statistical data.

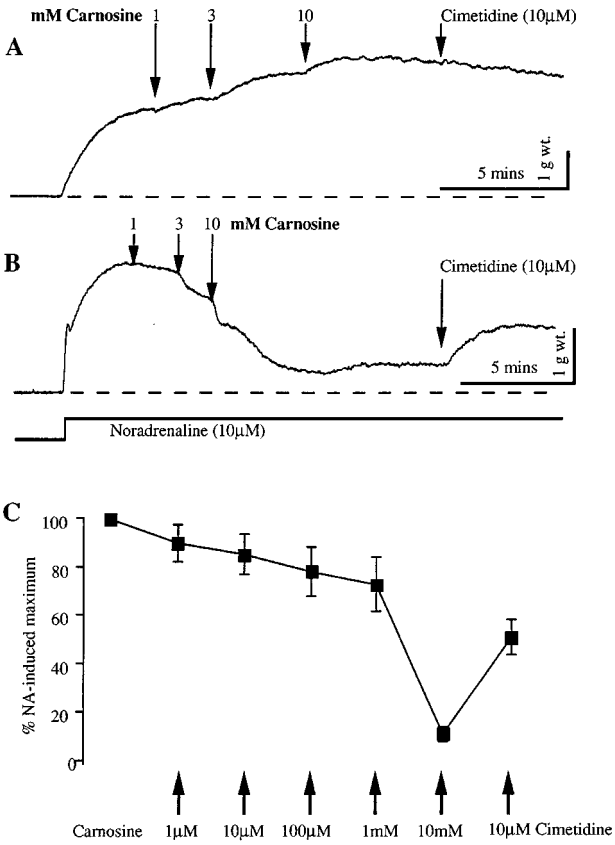

Figure 7 H_2-mediated relaxing effect of carnosine. Example traces of the isometric response of two saphenous vein rings to carnosine. As in Figure 6, the tissues were pre-activated by 10 μM NA and tension allowed to stabilize. Three levels of carnosine, followed by one of cimetidine were applied (as indicated above the traces), either in the absence (A) or the presence (B) of 10 μM mepyramine. (C) shows collected data (means $\pm$ s.e.mean, $n = 5$ to 7) for the response obtained in the presence of mepyramine (i.e. with the protocol in B). See text for statistical data.

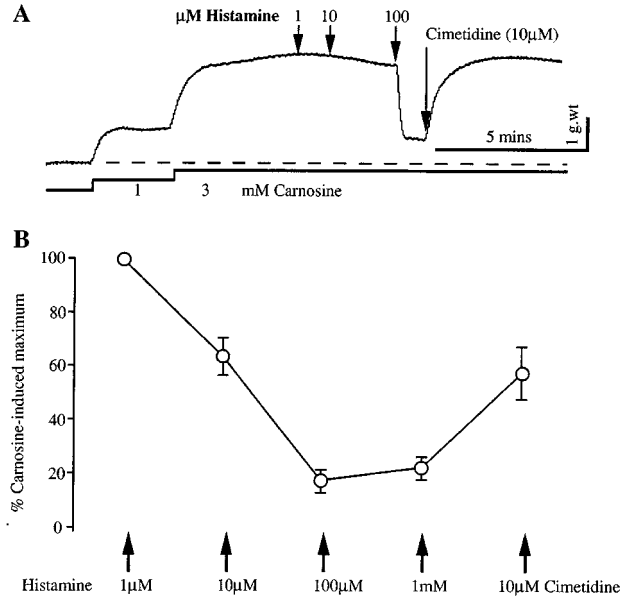

Figure 8 H_2-mediated relaxing effect of histamine in the presence of carnosine. (A) shows an example trace of the isometric response of a saphenous vein ring to histamine. The tissue was pre-activated by 3 mM carnosine and tension allowed to stabilize. Three levels of histamine, followed by one of cimetidine were applied (as indicated above the traces). (B) shows collected data (means $\pm$ s.e.mean, $n = 4$ to 12) for the responses obtained with the protocol in Figure 5A. See text for statistical data.

receptor (Wouters *et al*., 1985; Schotte & Leysen, 1988) and cross sensitivity between 5-HT_2 and H_1 sites has also been noted for [^{3}H]-mianserin (Peroutka & Snyder, 1981). Our initial conclusions that carnosine acts through a serotonergic receptor is, therefore, equivocal. We decided therefore, to seek more direct evidence for carnosine binding at the H_1-receptor by using a radioligand binding assay.

The use of [^{3}H]-mepyramine as a selective, high affinity antagonist ($K_d \sim 1$ nM) at H_1-receptors in brain and other tissues is well-documented (e.g. Hill *et al*., 1977, 1978) and it is still considered the tritiated ligand of choice for H_1-receptor studies. We chose to use guinea-pig cerebellum since the density of H_1-receptors here is high (the highest of all tissues studied), and conveniently, the density of 5-HT receptors is low (Garbarg *et al*., 1992). Zinc and other divalent cations have previously been reported to inhibit binding of [^{3}H]-mepyramine at H_1-receptors (Treherne *et al*., 1991) although the exact mechanism is unknown. Under our assay conditions, Zn inhibited [^{3}H]-mepyramine binding with a $\log IC_{50}$ of -4.20; this effect was taken into consideration by subtracting the value obtained for Zn alone in each individual assay performed. Non-specific binding to receptors other than H_1 was consistently low.

The results of the binding studies support those obtained in the functional studies in RSV. Carnosine on its own had very little effect on [^{3}H]-mepyramine binding, but in the presence of micromolar concentrations of Zn, binding was greatly potentiated and was saturable. However, we have taken our analysis of these results a stage further and re-plotted the data against concentration of Zn•Carn complex (which can be calculated for each concentration of Zn and carnosine). Results obtained with a wide range of total Zn and carnosine concentrations can thus be satisfactorily described by a single curve with $\log IC_{50}$ of -5.61. This affinity, measured directly at the receptor, is somewhat lower ($30\times$) than that previously observed for the contractile effect of Zn•Carn in isolated saphenous vein ($\log EC_{50}$ -7.13) as might be expected. The potency of histamine in competing for [^{3}H]-mepyramine binding has also been observed to be substantially less than its biological potency in some tissues (Chang *et al*., 1979). However, the potency of Zn•Carn is almost identical to that of histamine itself in the same binding assay ($\log IC_{50} - 5.54$) and the Hill coefficients are very similar (0.87 and 0.78 respectively). All of these results from the binding work, an entirely independent approach from the contractile studies, provide strong support for our contention that carnosine (as Zn•Carn) is a novel, potent agonist at the H_1 receptor.

The radioligand binding studies were primarily done to provide definitive evidence for Zn•Carn acting at the H_1-receptor in blood vessels. However, the fact that this work used cerebellum as the source of H_1-receptors also reveals the potential for Zn and carnosine interacting at this receptor in the central nervous system. Zinc is distributed throughout brain and Zn-dependent modulation of neuronal activity is increasingly recognized (Smart *et al*., 1994). Zinc influences central neurone excitability (through e.g. subtypes of glutamate and γ-aminobutyric acid (GABA) receptors) and is released from active hippocampal neurones (Assaf & Chung, 1984; Howell *et al*., 1984), resulting in local concentrations of up to 300 μM (Assaf & Chung, 1984). Since carnosine-rich areas in brain have also been identified (Sassoe-Pognetto *et al*., 1992, 1993) it is feasible that this substance will be able to

interact with Zn in the CNS. Although a co-transmitter or neuromodulator role for CNS carnosine has been suggested, interaction with Zn has only been speculated upon (e.g. Sassoe-Pognetto *et al*., 1992); in the light of our recent findings this merits re-evaluation. Thus, our observations reveal a previously unsuspected phenomenon whose relevance could extend beyond vascular smooth muscle.

The observation that carnosine and histamine appeared to have shared activity at the H_1 receptor begged two questions: First, whether Zn would potentiate the contractile responses to histamine in RSV; we have found that it does (O'Dowd & Miller, 1996 and unpublished observations). Second, whether carnosine, like histamine, could have H_2-mediated relaxation effects. Results show that, in the presence of mepyramine, carnosine does induce relaxation which is cimetidine-sensitive. However, its effect is much less potent than that of histamine, significant relaxation occurring only at 1 mM and above (compared with a 10 μM threshold for histamine). This is reflected in the lack of potentiation of the carnosine contractile response in the presence of cimetidine. Neither carnosine- nor histamine-induced relaxation was potentiated by the addition of Zn. Nevertheless, this evidence suggests that carnosine, like histamine, could have hyper- or hypotensive actions, depending upon the preponderance of H_1 or H_2 receptors throughout the cardiovascular system. (Preliminary observations on small, subcutaneous 'resistance' arterioles c. 200–300 μm in diameter by wire myography (see Mulvany & Aalkjaer, 1990 for details) reveal a Zn-potentiated, mepyramine-antagonized contractile effect of carnosine, which is thus not confined to large veins such as saphenous).

This may go some way towards explaining the hypotensive effect of carnosine *in vivo* (Mason & Binkley, 1931; du Vigneaud & Hunt, 1936); although histamine contracts many isolated vascular preparations, it too has a hypotensive effect *in vivo*, reflecting predominantly H_2-mediated relaxation (Powell & Brody, 1976). Histamine-induced relaxation in arterial preparations appears to occur *via* an endothelial H_1-receptor (Van De Voorde & Leusen, 1983), but this aspect has not yet been explored in relation to carnosine.

The observation that carnosine (at concentrations eliciting maximal or near-maximal contraction) could 'reveal' the H_2-mediated relaxation of histamine is consistent with the binding of the former to the H_1-receptor. Whether such an interaction might occur *in vivo* is open to speculation.

The cyclooxygenase inhibitor indomethacin, at a concentration which should inhibit prostaglandin synthesis, had no effect on the carnosine CRC. In contrast, the maximal contractile response of histamine was depressed by $27.2 \pm 8.3\%$ of control. A similar effect (30% depression) has been observed in isolated human saphenous vein (Schoeffter & Godfraind, 1989) and was attributed to indirect (H_1-mediated) release of prostanoids, probably from vascular endothelium. It is not clear why carnosine, if acting *via* the same receptor as histamine, apparently did not elicit this response and suggests that carnosine and histamine may not completely overlap in terms of receptor binding. This might imply a difference between smooth muscle and endothelial H_1 receptors, but is beyond the scope of the present report.

Studies with radiolabelled histidine have demonstrated a metabolic link between carnosine, carcinine (β-alanyl-histamine) and histamine (Flancbaum *et al*., 1990a). Carnosine is present in many histamine-rich tissues where, it has been suggested, it serves as a non-mast cell source of histamine (*via* histidine) during physiological 'shock' (Fitzpatrick *et al*., 1990). The view of carnosine as a functionally inert precursor for histamine may have to be re-assessed in light of the present findings. H_1 receptors are widespread in a variety of mammalian tissues (Hill, 1990) as is carnosine. For further discussion of the possible receptor-modulated physiological role for carnosine and its relationship with Zn, see O'Dowd *et al*. (1996).

In summary, the present study indicates that the vasoconstricting action of Zn•Carn in RSV is mediated *via* an H_1 (mepyramine-sensitive) receptor; the inhibitory effect of the serotonergic antagonists would appear to be coincidental because, as reported here (Figure 3) these agents antagonize histamine itself. However, until it has been demonstrated that ketanserin and methiothepin interact at [^{3}H]-mepyramine binding sites with comparable affinities to those determined from the functional studies, and in the absence of a suitable selective 5-HT_1-like antagonist, some involvement of the 5-HT_1-like receptor cannot be completely excluded. In addition to its constrictor action, carnosine has a less potent relaxing effect, probably *via* an H_2 (cimetidine-sensitive) receptor; this may help to explain the hypotensive action of carnosine *in vivo* in some species. Furthermore, the results show that Zn•Carn binds to CNS H_1-receptors, raising the possibility of a further, novel action *in vivo*.

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