# The dipeptide carnosine constricts rabbit saphenous vein as a zinc complex apparently via a serotonergic receptor

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1. The endogenous dipeptide carnosine ($\beta$-alanyl-L-histidine), at 0·1–10 mM, provokes sustained contractures in rabbit saphenous vein rings with greater efficacy than noradrenaline (NA).

2. The effects of carnosine are specific; anserine and homocarnosine are ineffective, as are carnosine's constituent amino acids histidine and $\beta$-alanine.

3. Maximum carnosine-induced tension is enhanced by Zn ions (e.g. to 127·5 $\pm$ 13·1 % of control at 10 $\mu$M total Zn concentration, $Zn_{tot}$) and the sensitivity to carnosine potentiated (mean [carnosine] required for half-maximal tension, $K_{1/2}$, reduced from 1·23 mM to 17·0 $\mu$M carnosine with 15 $\mu$M $Zn_{tot}$).

4. The dipeptide apparently acts as a zinc–carnosine complex. The effects of carnosine at concentrations of 1 $\mu$M to 10 mM in the presence of 1–100 $\mu$M $Zn_{tot}$, can be described as a unique function of the concentration of Zn–carnosine, with an apparent $K_{1/2}$ for the complex of $7·4 \times 10^{-8}$ M.

5. Contractures are reduced at low $[Ca^{2+}]$, unaffected by adrenoceptor antagonists, but can be blocked by serotonergic receptor antagonists including ketanserin and methiothepin.

6. Competition between albumin and carnosine for Zn ions, as might occur in plasma, can be demonstrated experimentally.

7. The mode of action of carnosine is virtually unique: a vascular muscle receptor apparently transduces the action of a dipeptide in the form of a metal chelate.

We have studied a range of actions of the histidyl dipeptide carnosine ($\beta$-alanyl-L-histidine), one of a group of endogenous compounds including anserine ($\beta$-alanyl-1-methyl-L-histidine) and homocarnosine ($\gamma$-aminobutyryl-L-histidine) (Miller, Lamont & O'Dowd, 1993). Carnosine occurs at high concentration in skeletal muscle (1–50 mM; Crush, 1970), is lower in cardiac muscle (*ca* 1 mM) (Miller *et al.* 1993) and in plasma is, in many cases, undetectable ($< 0·1$ $\mu$M by the most sensitive method to date, Dunnett & Harris, 1992). Several actions have been ascribed to carnosine: (a) it is the major organic pH buffer in skeletal muscle; (b) alone, or in combination with its relatives, it can have radical scavenging activity (e.g. MacFarlane, McMurray, Dargie, O'Dowd & Miller, 1991); (c) it is thought to be a neurotransmitter and/or a neuromodulator in the olfactory system (Sassoe-Pognetto, Cantino & Fasolo, 1992); (d) it can influence some enzymes of energy transduction, notably phosphorylase a and b (Boldyrev & Severin, 1990); (e) we have reported a $Ca^{2+}$-sensitizing action in skeletal and cardiac muscle (Miller *et al.* 1993); and (f) finally, it is known to have effects on vascular tone. However, the physiological role of carnosine remains unclear. Some

60 years ago it was first reported (Mason & Binkley, 1931; du Vigneaud & Hunt, 1936) that bolus injections of carnosine *in vivo* reduce blood pressure, although this action is unexplained. Here we report that carnosine, in the form of a Zn–carnosine complex, increases tone in isolated blood vessels by a receptor-mediated action. Preliminary results have been presented (O'Dowd, O'Dowd & Miller, 1995).

## METHODS

Vascular rings (2–3 mm long) from the lateral saphenous vein of the rabbit (New Zealand White, 2·2–2·4 kg, killed by I.V. overdose of sodium pentobarbitone, 120 mg kg$^{-1}$) were mounted for isometric tension recording in standard organ baths (10–15 ml volume) containing normal physiological saline solutions at 37 °C equilibrated with 95 % $O_2$–5 % $CO_2$ (for details see Daly, McGrath & Wilson, 1988). The saline solutions contained (mM): NaCl, 118·4; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; $NaHCO_3$, 24·9; $KH_2PO_4$, 1·2; and D-glucose, 11·1.

### Protocol

Preparations were mounted under an original resting tension of 1·5 g wt and allowed to relax. Final resting tension was between 0·3 and 0·5 g wt. After an equilibration period of 1 h, the bathing

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solutions were changed once and a further 1 h equilibration period was allowed before commencing the experimental regime. Where zinc acetate was used, it was added 5–10 min before the addition of carnosine. The same initial equilibration procedure was employed in experiments involving the antagonists prazosin, rauwolscine, methiothepin and ketanserin, except that during the second equilibration period the tissues were pre-exposed to a single dose of carnosine ($10^{-4}$ M) in order to minimize changes in sensitivity between first and second cumulative exposures to carnosine (see text in relation to Fig. 1C). Preparations were then cumulatively activated with carnosine ($10^{-7}$ to $3 \times 10^{-3}$ M). After washout, preparations were incubated for 30 min with the antagonist and the cumulative exposure to carnosine was repeated. One preparation in each set had no antagonist added and acted as a time control (providing data, for example, for Fig. 1C). The antagonist experiments were all done in the presence of 5 $\mu$M Zn. Contractures were evoked by applying the agonist from stock (at pH 7·4 where relevant) at 0·1 % of bath volume. Drugs were added in the same way, dissolved in water. Tension was allowed to stabilize (see Fig. 1A) before agonist concentration was raised further. Tension signals were recorded (usually sampled at 5 Hz) and analysed using MacLab8 (AD Instruments, Hastings, UK) hardware and software. Generally, several preparations from the same animal were assayed in parallel. Absolute steady-state tension at each concentration of agonist was measured.

Dose–response curves were fitted to the Hill equation:

$$\frac{\text{Tension}}{\text{Tension}_{\text{max}}} = \frac{[\text{carnosine}]^{n_{\text{H}}}}{K_{\frac{1}{2}} + [\text{carnosine}]^{n_{\text{H}}}},$$

with the maximum constrained to unity or derived from the best-fit as appropriate. Mean amplitudes were normalized to the mean maximum tension (tension$_{\text{max}}$) achieved with 10 mM carnosine in nominally Zn-free solutions or to their own mean maximum tension. Individual best-fit curves were fitted using the Levenberg–Marquardt algorithm (with Igor Pro software, WaveMetrics Inc., Lake Oswego, OR, USA). The curves are conveniently characterized by the values of log $K_{\frac{1}{2}}$ (log$_{10}$ of the concentration of agonist giving half-maximal tension) and the Hill coefficient, $n_{\text{H}}$. Curves illustrated are plotted using the mean values obtained for these individual best-fit parameters, with the normalized tension responses obtained at each concentration of the agonist plotted as the means ± S.E.M. to indicate the variability of responses.

Zinc and other ions tested were added from 1–100 mM stocks of the acetate or chloride salt in distilled water in volumes not exceeding 0·1 % of bath volume. (80 mM is at or near the solubility limit for zinc acetate at least, as confirmed by spectrophotometric measurements on stock samples.) Chemicals were from Sigma, except ketanserin (Janssen Pharmaceutica, Beerse, Belgium). Albumin (99 % pure, essentially globulin free; Sigma) was from rabbit.

The concentration of the Zn–carnosine complex reported in Fig. 3 was calculated using a multi-ligand, multi-ion program (REACT, written by Dr G. L. Smith, University of Glasgow, UK). The program was developed, according to principles described elsewhere (Smith & Miller, 1985), for work with chemically skinned muscle fibres. The relevant stoichiometric affinity constants were employed (Sillén, Martell, Hogfeldt & Smith, 1974; Pettit & Powell, 1993). Values for the association constants ($K$) for carnosine (L), LH–Ca and LH–Mg, are not available in the literature, but may be estimated pro rata to approximately 1·0 (log $K$). However, trial calculations revealed that the concentration of Zn–carnosine was

hardly affected by these reactions. The same tabulation was used for the relevant EDTA constants. Solution pH was assumed to be 7·40 (bicarbonate/$CO_2$ buffered).

Data are quoted as means (± S.E.M for accumulated and derived parameters) with $n$ = number of preparations and the number of animals given in parentheses. The significance of any differences between these parameters for individual test and control preparations was assessed by Student's $t$ test, with significance assumed at $P \leqslant 0.05$.

# RESULTS

We found in preliminary work that a small proportion of vessel preparations respond to 10–100 $\mu$M carnosine with a sustained contracture of widely varying amplitude. Additionally, we found a small, but significant, tendency of carnosine to potentiate contractures induced by noradrenaline (NA). Since skeletal muscle intracellular carnosine concentration can exceed 10 mM, we considered that high concentrations might occur at certain times in the extracellular space. We therefore tested carnosine up to 10 mM and found that contractures are consistently evoked at 0·1 mM or more. Tension develops rapidly and is well sustained (Fig. 1A). The concentration–response curve for carnosine (obtained cumulatively) is shown in Fig. 1B. A repeat of the cumulative exposure to carnosine reveals that the preparation becomes slightly desensitized. Figure 1C shows accumulated data for the first and second runs with carnosine. The log $K_{\frac{1}{2}}$ increased from −4·48 to −4·16 for these twelve preparations. This behaviour is typical agonist desensitizing as observed in isolated blood vessels with many agonists and is taken into account in our protocols where comparisons have been made between the second sets of responses. With the exception of this small desensitization, the responses are reproducible and reversible. There is some small variation in 'control' sensitivity to carnosine between groups of experiments (compare for example Fig. 1 with Fig. 4), so 'control' data are always those relevant to the group in question. Contractures do not require functional endothelium (removed by 'rubbing' the vessel). With the endothelium intact, acetylcholine promotes rapid, full relaxation of the carnosine-induced contractures (CICs). The closest chemical relatives to carnosine, anserine and homocarnosine, are ineffective (up to 1 mM), as are histidine (up to 1 mM) and $\beta$-alanine (up to 10 mM), carnosine's constituent amino acids.

The maximum tension evoked by carnosine is approximately double that for an optimal concentration of noradrenaline (NA; see Fig. 1B legend) irrespective of at which stage of the experiment NA is tested. The dose of NA used (30 $\mu$M), reliably evokes the maximum response in this tissue under the present conditions (see e.g. Daly *et al.* 1988). Addition of carnosine at the peak of a maximal NA-induced contracture affords a direct demonstration that tension is nearly doubled by carnosine (Fig. 1D).

We found that 20 $\mu$M EDTA (added to chelate contaminant iron ions and intended to minimize NA oxidation) greatly attenuates the CIC (Fig. 1$B$); 100 $\mu$M EDTA attenuates it still further (data not shown), suggesting that a trace heavy metal ion is essential to the action of carnosine. (20 $\mu$M EDTA has only negligible effects on $[Ca^{2+}]$ or $[Mg^{2+}]$ in the saline solutions.) Carnosine binds a number of ions including $Cu^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ (Sillén *et al.* 1974). We tested each of these ions in the presence of carnosine. At concentrations of up to 10 $\mu$M $[\text{metal ion}]_{tot}$, only Zn facilitated carnosine's action. (Zn ions alone, or the accompanying anion, acetate, do not induce contracture without carnosine being present.) We studied the effects of 15 $\mu$M Zn, the total concentration in mammalian plasma (Lentner, 1981, 1984), in more detail (Fig. 1$B$) including Zn reducing the mean log $K_{\frac{1}{2}}$ from $-2\cdot92 \pm 0\cdot04$ ($n = 29$, (11)), to $-4\cdot79 \pm 0\cdot06$, ($n = 12$, (7)). The threshold for the action of carnosine was thus reduced to near 1 $\mu$M and maximum tension was achieved at 100 $\mu$M. The rightward shift produced by EDTA is partially reversed by adding 15 $\mu$M Zn (Fig. 1$B$).

### Characterization of Zn–carnosine interaction

We hypothesized that carnosine might act in the form of a Zn–carnosine complex. The reduction in effectiveness of carnosine by EDTA in nominally Zn-free conditions could
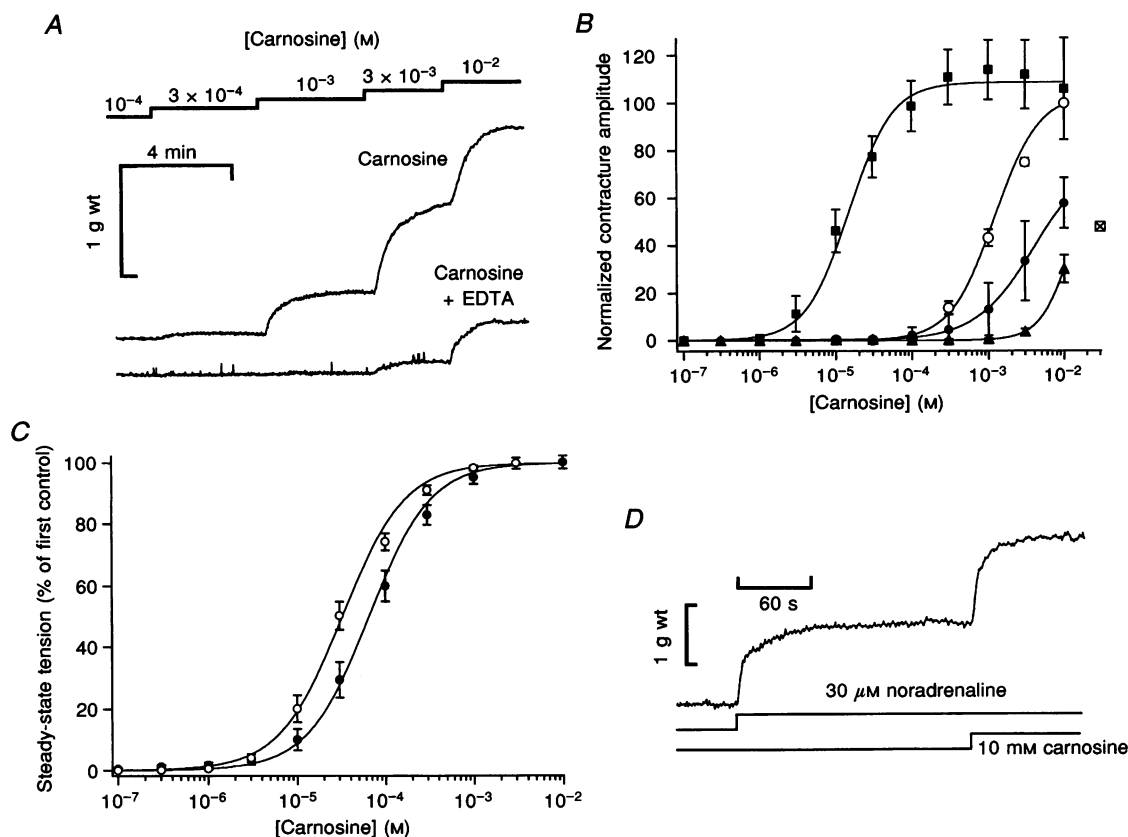
**Figure 1. Responses of saphenous vein to carnosine**

*A*: upper trace, isometric tension responses in rabbit saphenous vein, carnosine was added cumulatively; lower trace, the same protocol with 20 $\mu$M EDTA. *B* shows contracture amplitude (steady-state tension) *vs.* carnosine concentration as a percentage of mean response to 10 mM carnosine (O; $n = 29$, (12); 100% = $3\cdot0 \pm 0\cdot10$ g wt). Symbols as follows: ⊠, maximum response to 30 $\mu$M NA alone (s.e.m. smaller than the symbol), results obtained without EDTA or NA uptake inhibitors such as cocaine in separate experiments ($n = 29$, (9)). ▲, 20 $\mu$M EDTA (from pH 7·4 stock; $n = 6$, (5)). ■, 15 $\mu$M Zn (as zinc acetate; $n = 6$, (6)). ●, 20 $\mu$M EDTA + 15 $\mu$M Zn ($n = 6$, (6)). log $K_{\frac{1}{2}}$ values were: $-4\cdot79 \pm 0\cdot06$ (15 $\mu$M Zn); $-2\cdot92 \pm 0\cdot04$ (control); $< -2\cdot0$ (EDTA); $-1\cdot90$ (EDTA + Zn); Hill coefficients were $1\cdot20–1\cdot50$. (All $K_{\frac{1}{2}}$ values were significantly different from 'control' values and one another.) *C*, cumulative curve of steady-state tension against carnosine concentration illustrating a 'desensitization' to carnosine between first (O) and second runs (●) (using the 'antagonist' protocol described in Methods, thus with 5 $\mu$M Zn present). Tensions were normalized relative to the maximum for the first run for each preparation. Absolute maximum tension was unchanged (to $100\cdot06 \pm 1\cdot91$%; $n = 7$, (12); n.s.) but log $K_{\frac{1}{2}}$ increased significantly from $-4\cdot49 \pm 0\cdot07$ (O) to $-4\cdot18 \pm 0\cdot09$ (●); Hill coefficients were $1\cdot21$ and $1\cdot23$ for first and second runs, respectively. *D*, isometric tension trace illustrating that greater maximum force was achieved with 10 mM carnosine than with 30 $\mu$M noradrenaline (see text for details).
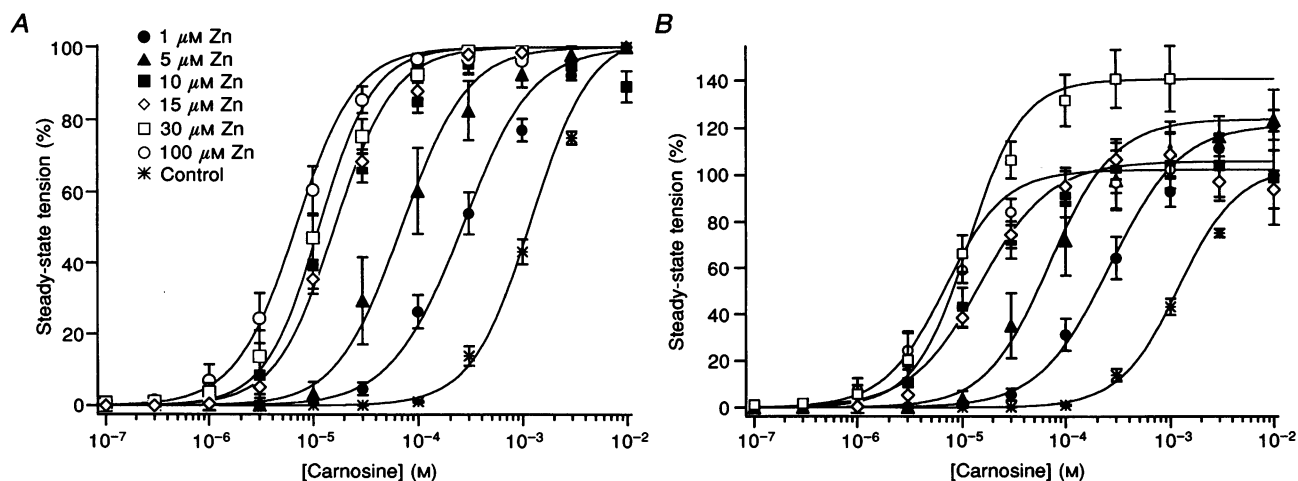
**Figure 2. Effects of Zn on responses to carnosine**

Concentration–response curves with six Zn concentrations. Symbols are defined in $A$ for both panels. Results from 4–29 preparations from 2–15 animals. $A$, responses normalized to their own maxima; mean $\log K_{1/2}$ values for carnosine were: $-3.59 \pm 0.08$, $-4.16 \pm 0.16$, $-4.83 \pm 0.09$, $-4.79 \pm 0.06$, $-4.94 \pm 0.09$, and $-5.17 \pm 0.10$ for 1, 5, 10, 15, 30 and 100 $\mu$M Zn, respectively; control $\log K_{1/2}$ was $-2.92 \pm 0.04$ (the curve for 15 $\mu$M Zn has been omitted from the figure for clarity). Hill coefficients ranged from $1.66$ to $1.28$. $B$, the same data expressed relative to the maximum response observed at 10 mM carnosine (no added Zn). In $A$ and $B$, $\log K_{1/2}$ values for all curves were significantly different from control values ($P \ll 0.0001$) and one another ($P < 0.03$ or better), except 1 $vs.$ 5 $\mu$M Zn curves ($P < 0.08$). In $B$, 1, 5 and 30 $\mu$M maxima were significantly greater than control ($P < 0.001$).
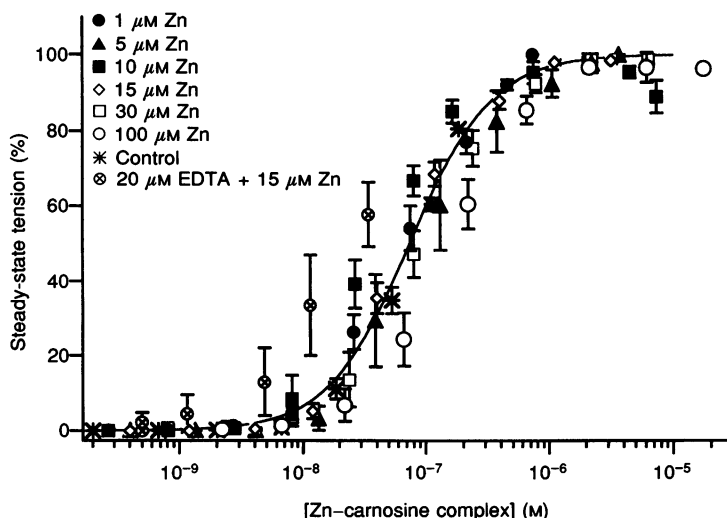


**Figure 3. Tension responses plotted as a function of the Zn–carnosine complex concentration**

Transformation of data (mean steady-state tension for 4–29 preparations from 2–15 animals) obtained with 6 different added Zn concentrations. Values for concentration of Zn–carnosine were calculated as described in Methods. Symbols are defined in the figure. Results for control (nominally Zn-free) solutions were calculated assuming $0.25$ $\mu$M $Zn_{tot}$ 'contamination'. The curve is fitted with a $\log K_{1/2}$ of $-7.13$ ($= 7.4 \times 10^{-8}$ M) for Zn–carnosine and a Hill coefficient of $1.34$. (Limited zinc acetate solubility (see Methods) means that the nominal 100 $\mu$M Zn solutions were actually nearer 80 $\mu$M and the calculation was made on that assumption.)

be explained if 'contamination' levels of Zn were chelated by EDTA, preventing Zn–carnosine formation. This hypothesis was tested by using a range of Zn concentrations. The results obtained with several concentrations of Zn (1–100 $\mu$M added) are summarized in Fig. 2 (data normalized to their own maximum in Fig. 2A and to the maximum in the nominally Zn-free solutions with 10 mM carnosine in Fig. 2B).

Figure 2A reveals a simple leftward shift of the dose–response curve with as little as 1 $\mu$M Zn. At 100 $\mu$M, the highest concentration tested (see Methods), Zn increases apparent carnosine sensitivity above that achieved at 15 $\mu$M Zn by a further 2·3-fold (log $K_{\frac{1}{2}}$ values and Hill coefficients are given in the legend).

Inspection of the control curve (carnosine in nominally Zn-free medium) indicates that even at 10 mM carnosine, absolute maximum tension levels are not maximal. Figure 2B shows that maxima are substantially greater at intermediate Zn concentrations ($P < 0·001$, except at 10 and 15 $\mu$M, e.g. 123·8 $\pm$ 5·4% at 5 $\mu$M, 140·7 $\pm$ 14·2% at 30 $\mu$M, compared with 103·7 $\pm$ 0·94% for carnosine alone (unconstrained best-fit curves); 100% = 3·00 $\pm$ 0·10 g wt). The lower maxima achieved with 100 $\mu$M Zn (102·5 $\pm$ 17·8%) might reflect a non-specific inhibitory effect of Zn ions at such high concentration.

The concentration of the Zn–carnosine complex in these media can be calculated (see Methods). The tension data from Fig. 2A are replotted in Fig. 3 against the calculated Zn–carnosine complex concentration.

This transformation reveals that a unique curve satisfactorily relates [Zn–carnosine] in the range of $10^{-8}$ to $10^{-6}$ M to contracture amplitude, regardless of the absolute concentrations of either carnosine or added zinc.

Can the results obtained in nominally Zn-free conditions, or in the presence of EDTA be explained on this basis, assuming a single 'contamination' level of Zn (either in the media or derived from the preparation)? Results obtained in nominally Zn-free (i.e. 'control') solutions fit the curve well (Fig. 3) if as little as 0·25 $\mu$M Zn contamination is assumed (atomic absorption spectrophotometry of our solutions indicates approximately 0·5 $\pm$ 0·15 $\mu$M Zn). The extent to which tension is restored by adding Zn to EDTA can also be described, at least approximately, as illustrated in Fig. 3. However, there is a residual response to 10 mM carnosine in the presence of EDTA (20 $\mu$M, Fig. 1 and even 100 $\mu$M, data not shown).

*In vivo*, in the circulating blood, most Zn ions are bound to the plasma protein albumin. We tested the ability of albumin to antagonize the action of carnosine (Fig. 4). Using the physiological plasma albumin concentration (45 g l$^{-1}$ in man) was impractical because of solution frothing. However, 4·5 g l$^{-1}$ albumin greatly reduced the control responses (log $K_{\frac{1}{2}}$ increased from $-2·98 \pm 0·16$ ($n = 6$, (2)) to $-1·84 \pm 0·16$ for carnosine ($n = 5$, (2))), whereas 0·45 g l$^{-1}$ (log $K_{\frac{1}{2}}$ was $-2·76 \pm 0·23$ ($n = 4$, (2))) proved virtually ineffective. These latter two concentrations bracket those in extracellular fluids such as cerebrospinal fluid ($ca$ 0·7 g l$^{-1}$; Lentner, 1981, 1984). Albumin (at the higher concentration) reduces the effectiveness of 15 $\mu$M Zn (the total plasma
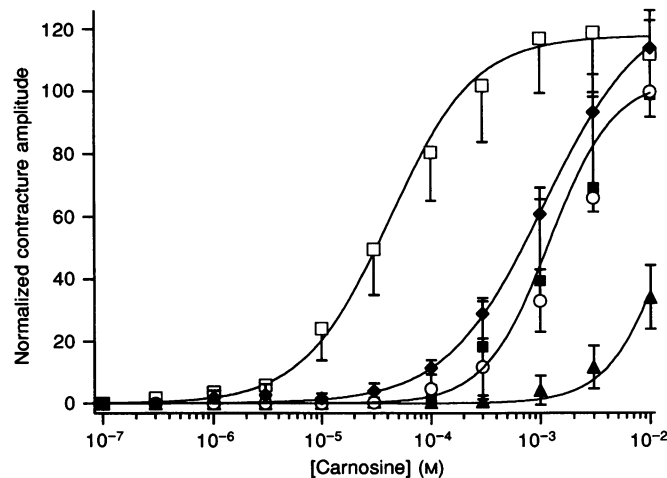


**Figure 4. Effects of albumin on cumulative carnosine-induced contractures**

Steady-state isometric tension responses: control (O) log $K_{\frac{1}{2}}$ of $-2·98 \pm 0·16$ was increased (n.s.) to $-2·76 \pm 0·23$ and $-1·84 \pm 0·16$ by 0·45 g l$^{-1}$ (■, n.s.) and 4·5 g l$^{-1}$ albumin (▲, $P < 0·01$), respectively. Addition of 15 $\mu$M Zn significantly increased the sensitivity to carnosine + albumin, reducing log $K_{\frac{1}{2}}$ to $-4·38 \pm 0·18$ and $-2·97 \pm 0·05$ in 0·45 g l$^{-1}$ (□) and 4·5 g l$^{-1}$ albumin (◆), respectively. Numbers of preparations and animals are given in the text.
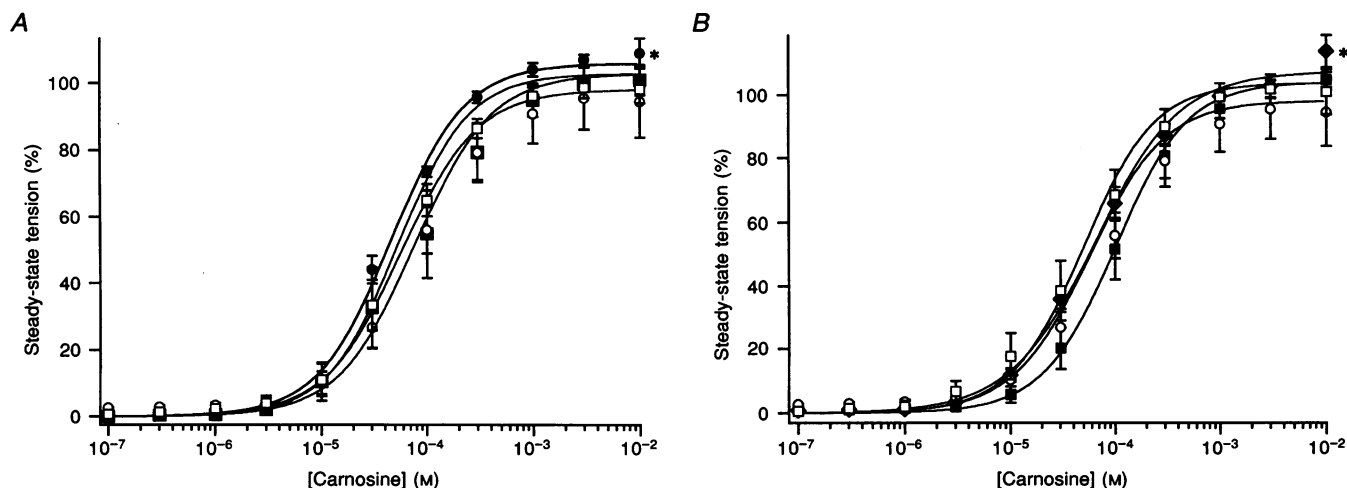
**Figure 5. No effect of $\alpha$-adrenergic antagonists on carnosine-induced contractures**

Lack of antagonism by prazosin ($A$) and rauwolscine ($B$) on cumulative carnosine-induced contractures (5 $\mu$M Zn added to all solutions) using the standard antagonist protocol described in Methods. $A$, prazosin concentrations were: $10^{-8}$ (●; $n = 3$, (3)); $10^{-7}$ (■; $n = 6$, (6)); and $10^{-6}$ M (□; $n = 6$, (6)). Rauwolscine concentrations were: $10^{-7}$ (■); $10^{-6}$ (□); and $10^{-5}$ M (◆); $n = 5$, (5) for all concentrations. Control (○) log $K_{\frac{1}{2}}$ was $-4\cdot25 \pm 0\cdot17$ for carnosine. Differences between curve parameters were not significant ($P > 0\cdot05$, except the greater maximum achieved with $10^{-8}$ M prazosin and $10^{-5}$ M rauwolscine).
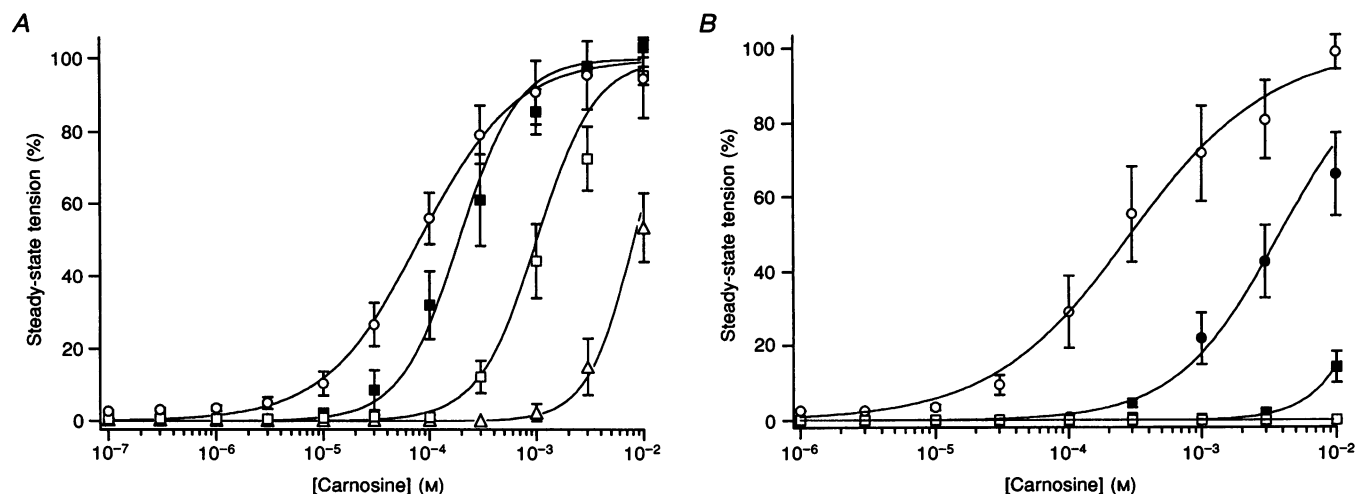


**Figure 6. Inhibition of carnosine-induced contractures by serotonergic antagonists**

Effect of the serotonergic antagonists ketanserin ($A$) and methiothepin ($B$) on carnosine-induced contractures (5 $\mu$M Zn added to all solutions) using the antagonist protocol described in Methods. $A$, ketanserin concentrations were $10^{-7}$ (■), $10^{-6}$ (□) and $10^{-5}$ M (△) with control (○); $n = 4$, (4) for all concentrations. $B$, methiothepin concentrations: $10^{-8}$ (●; $n = 8$, (5)); $10^{-7}$ (■; $n = 8$, (7)); and $10^{-6}$ M (□; $n = 7$, (6)); control, ○ ($n = 10$, (7)). For ketanserin, log $K_{\frac{1}{2}}$ rose significantly from $-4\cdot25 \pm 0\cdot17$ (control), to $-3\cdot72 \pm 0\cdot18$, $-2\cdot99 \pm 0\cdot14$ and $-2\cdot46 \pm 0\cdot04$ for $10^{-7}$, $10^{-6}$ and $10^{-5}$ M, respectively. All log $K_{\frac{1}{2}}$ values were significantly different from one another as well as from control values. Mean Hill coefficients were $1\cdot19$, $1\cdot57$, $1\cdot59$ and $1\cdot80$ for control and $10^{-7}$, $10^{-6}$ and $10^{-5}$ M, respectively. For methiothepin, log $K_{\frac{1}{2}}$ rose from $-3\cdot18 \pm 0\cdot07$ (control) to $-2\cdot45 \pm 0\cdot12$ and $-1\cdot61 \pm 0\cdot12$ for $10^{-8}$ and $10^{-7}$ M, respectively. Hill coefficients were $1\cdot35$, $1\cdot13$ and $1\cdot5$ for $10^{-8}$, $10^{-7}$ and $10^{-6}$ M, respectively. Carnosine-induced contractures were completely abolished with $10^{-6}$ M methiothepin.

concentration) in potentiating carnosine's effects (log $K_{1/2}$ is increased from $-4\cdot79 \pm 0\cdot06$ (see Fig. 1) to $-2\cdot97 \pm 0\cdot05$ ($n = 4$, (2))).

### Receptor transduction of carnosine's action?

CIC amplitude and form was affected by reducing extracellular [$Ca^{2+}$] from 2·5 to 0·5 mM or lower ($n = 6$, (2)); amplitude was reduced (tension was 61 % or less at 0·1 mM $Ca^{2+}$ or less; $n = 7$, (3)) and contractures were less well sustained (at $< 0\cdot1$ mM) and abolished in nominally zero $Ca^{2+}$ ($n = 3$, (2)) but returned when [$Ca^{2+}$] was restored to normal.

The speed and selectivity of action of carnosine (or apparently Zn–carnosine) suggested a receptor-mediated pathway. We have made a preliminary analysis by assessing the ability of receptor antagonists to block or reverse CICs. Several proved effective, but some only at high concentrations when they are less selective. Thus, phentolamine, an $\alpha_1$- and $\alpha_2$-adrenoreceptor blocker, relaxes the CIC (by more than 90 %), but only above $10^{-5}$ M ($n = 6$, (4)).

Figure 5 shows results obtained with the standard pre-exposure antagonist protocol (see Methods). Figure 5A shows the effects of the $\alpha_1$-adrenoceptor blocker prazosin ($10^{-8}$ to $10^{-6}$ M) and Fig. 5B shows the effects of the $\alpha_2$-adrenoceptor blocker rauwolscine ($10^{-7}$ to $10^{-5}$ M). Neither agent significantly reduces sensitivity to carnosine (see figure legend for details). In contrast, CICs prove sensitive to several 5-HT antagonists at $10^{-9}$ to $10^{-6}$ M, for example ketanserin and methiothepin. Ketanserin, a 5-HT$_2$ antagonist with some $\alpha_2$-antagonist activity, produced a significant, concentration-dependent rightward shift of the carnosine response curve at $10^{-7}$ to $10^{-5}$ M, with no associated alteration in the maximum response (Fig. 6A). Methiothepin, a potent (but non-selective) antagonist at '5-HT$_1$-like' receptors (Hoyer *et al.* 1994), produced a large rightward shift in the carnosine response curve at $10^{-8}$ M and complete inhibition of the response by $10^{-6}$ M (Fig. 6B).

All these antagonist tests were done with 5 $\mu$M Zn added, as described in the Methods, to ensure a higher initial sensitivity to carnosine (see Fig. 2).

## DISCUSSION

We have demonstrated that the endogenous histidyl dipeptide carnosine can induce large sustained contractures (CICs) in vascular smooth muscle. Blockage by the divalent cation chelator EDTA and potentiation by adding Zn, but none of several other transition metals, suggest the effect is attributable to the formation of a Zn–carnosine complex. Figure 3 shows that the results obtained with a very wide range of concentrations of Zn and carnosine can be reduced satisfactorily to a single curve if they are expressed in terms of the concentration of the complex. (One might dismiss the leftward deviation for the curve fitting the Zn + EDTA results, given the large number of ionic interactions which determine the concentration of Zn–carnosine.) On this basis,

Zn–carnosine is the active species and is effective with an apparent log $K_{1/2}$ of $-7\cdot13$ ($= 7\cdot4 \times 10^{-8}$ M). However, there is a residual response to 10 mM carnosine in the presence of EDTA (20 $\mu$M, Fig. 1 and even 100 $\mu$M, data not shown) where Zn–carnosine is calculated to be at least an order of magnitude lower than $10^{-8}$ M (i.e. far to the left in Fig. 3). We must conclude that a fraction of the response to carnosine is not easily attributable to interactions involving contaminating Zn ions, although carnosine might interact with tissue-bound Zn inaccessible to EDTA.

A circulating carnosinase ensures that general plasma concentrations of the dipeptide remain low (Gardner, Illingworth, Kelleher & Wood, 1991). However, nothing is known about interstitial levels, local release, uptake or synthesis in relation to vascular smooth muscle. In circumstances where extracellular carnosine concentration might rise, e.g. for muscle vasculature during or after exercise, or the mesenteric beds after carnosine-rich white meat ingestion, concentrations achieved remain to be established. However, because the maximum tensions that Zn–carnosine can evoke in vascular smooth muscle are so very large, even low levels of the conjugate could be physiologically relevant. Saphenous vein drains skeletal muscle and might be exposed to higher concentrations of carnosine than some other vessels. We have found that saphenous artery behaves in a very similar way in response to carnosine. Carnosine induces contracture in several porcine vessels (also potentiated by Zn; V. Wilson and colleagues, personal communication). Rat aorta, however, proved totally insensitive, even when Zn ions were added (data not shown).

*In vivo* competition with plasma albumin for Zn ions probably keeps circulating Zn–carnosine concentration near or below the threshold for vasoactivity; Fig. 4 demonstrates such competition experimentally. However, where albumin is largely excluded (notably interstitial and cerebrospinal fluids) Zn–carnosine formation could assume more importance.

The action of carnosine through a vascular muscle receptor is thus virtually unique in two ways: it is a dipeptide agonist, effective in the form of a metal chelate. The only analogous action of a dipeptide is claimed for N-acetyl-aspartylglutamate (NAAG), which acts as a specific, low potency agonist at the N-methyl-D-aspartate subclass of ionotropic acidic amino acid receptors (Valivullah, Lancaster, Sweetnam & Neale, 1994). We know of no evidence for a metal chelate, as opposed to the ion itself (see below), acting through a receptor.

Tests of a variety of known receptor agonists and antagonists against CICs indicate a receptor-mediated action, which is not likely to be adrenergic (e.g. Fig. 5). The results, including a possible contribution of extracellular calcium, are consistent with other reports of an atypical '5-HT$_1$-like' receptor, which mediates 5-HT-induced contraction in rabbit saphenous vein (e.g. Martin &

McLellan, 1990; Van Heuven-Nolsen, Tysse Klasen, Luo & Saxena, 1990). At present, no selective antagonists exist for this receptor, but it is known to be very sensitive to methiothepin ($pA_2 > 7$; Hoyer et al. 1994), consistent with the present results (Fig. 6B). In addition, it has some characteristics of the 5-HT$_2$ receptor e.g. sensitivity to ketanserin (Hoyer et al. 1994), and so does not fit the currently recognized serotonergic receptor classification. Intriguingly, however, whilst $\alpha$-methyl-5-HT provokes contractures, 5-HT itself is only marginally effective in rabbit saphenous vessels under our conditions. Perhaps this indicates a receptor type for which Zn–carnosine is the natural agonist, and for which 5-HT sensitivity is of minor physiological relevance. Alternatively, the Zn–carnosine receptor might be a completely novel type with only coincidental sensitivity to the spectrum of antagonists used so far. A more detailed pharmacological analysis will be required to characterize the receptor unequivocally.

Our findings might have implications for other systems where both carnosine and Zn are present. The formation of a Zn–carnosine complex could be significant in brain (see above), where there are carnosine-rich regions and local Zn concentrations can reach 300 $\mu$M (Assaf & Chung, 1984; Sassoe-Pognetto et al. 1992). $Zn^{2+}$-dependent modulation of neuronal activity is increasingly recognized (Smart, Xie & Krishea, 1994). $Zn^{2+}$ influences central neurone excitability (through e.g. subtypes of glutamate and GABA receptors) and is released from active hippocampal neurones (Assaf & Chung, 1984; Howell, Welch & Frederickson, 1984). Although a cotransmitter or neuromodulator role for central nervous system carnosine has been suggested, interaction with Zn has only been speculated upon (e.g. Sassoe-Pognetto et al. 1992); in the light of the present observation this merits re-evaluation. Homocarnosine (GABA-L-histidine), a dipeptide closely related to carnosine and present in several brain regions, is only slightly vaso-constrictive in rabbit saphenous vein even when potentiated by Zn ions; data not shown. In addition, the ability of carnosine to bind with Zn might affect the activity of Zn-containing metalloproteins such as angiotensin- and endothelin-converting enzymes (present in the interstitial space) and the Cu–Zn form of superoxide dismutase. Inhibitors of angiotensin-converting enzyme, some of which act by a Zn binding mechanism (Zanchi, Nussberger, Criscuoli, Capone & Brunner, 1994), are arguably the most significant recent development in the management of high blood pressure. Thus, our observations reveal a previously unsuspected endogenous agonist–receptor system whose relevance could extend beyond vascular smooth muscle.

The effects we have described here reveal that carnosine acts, apparently through some form of serotonergic-like receptor, as a Zn chelate to initiate contraction. These results obviously do not explain the transient blood pressure reduction reported previously in vivo for cat and dog as well as rabbit (see Introduction). However, the action of vaso-active compounds in vivo is notoriously complex and varied

since more than one receptor subtype can be involved. Thus, for carnosine, any differential action on various vascular beds and possible central actions of the agonist on blood pressure cannot be excluded, at present. Although we have established here that carnosine has the capacity for profound effects on vascular smooth muscle tone, the precise physiological relevance of this phenomenon remains to be established.

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