Volume 329, number 3, 249–252 FEBS 12899 August 1993
© 1993 Federation of European Biochemical Societies 00145793/93/$6.00

Generation of substance P carbamate in neutral aqueous solution

Relevance to inflammatory joint diseases

Adrian J. Davis[a], Geoffrey E. Hawkes[a], Peter R. Haycock[a], Paul O'Brien[a], Bruce L. Kidd[b], Paul I. Mapp[b], Declan Naughton[b] and Martin Grootveld[b]

[a]*Department of Chemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, UK* and [b]*Inflammation Research Group, The London Hospital Medical College, 25–29 Ashfield Street, London, E1 2AD, UK*

Received 7 July 1993

High-field proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy has been employed to evaluate the formation of substance P carbamate in aqueous solution. Equilibration of substance P with physiologically relevant concentrations of bicarbonate (2.50×10^{-2} mol·dm^{-3}) at pH 7.00 generated a new multiplet signal centred at 4.13 ppm in its NMR spectrum, characteristic of the α-proton of peptide carbamate species. High-field ^{1}H NMR spectroscopy also demonstrated that the model dipeptide, Arg–Gly, formed a carbamate in neutral aqueous solutions containing 2.50×10^{-2} mol·dm^{-3} HCO_3^-. The physiological significance of these results is discussed in view of the central roles of vasoactive neuropeptides in human joint diseases and the hypercapnic environment of the inflamed rheumatoid joint.

Substance P; Neuropeptide; Carbamate; ^{1}H NMR; Inflammatory joint disease; Hypercapnic environment

1. INTRODUCTION

Substance P is a member of the tachykinin family of peptides and is localised within small primary sensory neurones throughout the body [1]. Currently, there is strong evidence to suggest that it serves to regulate the inflammatory response to a variety of stimuli [2]. Moreover, substance P interacts with a specific NK-1 receptor present on several cell types, including vascular smooth muscle and endothelial cells. Intra-dermal injections of substance P into human skin result in hyperalgesia, vasodilatation and plasma extravasation [3]. It also appears to play a wider role in immunoregulation, and in vitro studies have shown significant interactions with neutrophils, lymphocytes, macrophages and synoviocytes [4].

Recent reports indicate that human and experimental joint diseases have an important neurogenic component in which substance P plays a pivotal role [4]. Injections of substance P into minimally involved joints increases the severity of disease therein, and substance P antagonists significantly inhibit the early articular responses to inflammatory agents, such as carrageenin.

For many years it has been recognised that impairment of the vascular supply to the inflamed rheumatoid joint and/or an elevation in its metabolic rate gives rise to the markedly abnormal metabolic status of the intra-articular environment. Previous investigations of the physiological status of the inflamed joint have established an extremely low synovial fluid oxygen tension [5,6], increased carbon dioxide tension, diminished glucose concentrations, substantially elevated lactate levels and an associated acidosis [7–11]. Indeed, Falchuck et al. [7] have previously demonstrated that joints exhibiting severe microvascular destruction in the synovial membrane had the lowest pO_2, were substantially hypercapnic (high pCO_2), and contained high concentrations of lactate.

In view of the hypercapnic environment of the inflamed rheumatoid joint, it is conceivable that certain neuropeptides form carbamate species in this environment. Such carbamate production may play an important role in influencing the biological activity of neuropeptides, e.g. the modulation or elevation of their capacity to bind to protein receptors located on cellular membranes. The generation of amino acid, peptide and protein carbamates from the reaction of CO_2 with a free amino functional group (Eq. 1) in moderately basic solution has been known for many years [12–14] and is a phenomenon that has stimulated much biochemical interest since carbamate formation by

$$R\text{-}NH_2 + CO_2 = R\text{-}NHCO_2^- + H^+ \quad (1)$$

polypeptides is an important mechanism involved in the transport of CO_2 from respiring tissues [14,15]. Indeed, considerable quantities of CO_2 are bound to haemoglobin and plasma proteins in this manner [15], and the

Correspondence address: M. Grootveld, Inflammation Research Group, The London Hospital Medical College, 25–29 Ashfield Street, London, E1 2AD, UK.

dipeptide glycylglycine has been employed as a model for these reactions [13,16].

Our interest in the pathological roles of substance P in inflammatory joint diseases has led us to investigate the nature and level of carbamate formation for this vasoactive undecapeptide in neutral aqueous solution using high-field proton (^1H) nuclear magnetic resonance (NMR) spectroscopy. Proton NMR spectroscopy is a technique which is particularly suited to the study of carbamate production since the assignment of amino acid α-carbamate resonances is facile [17]. Moreover, it should also be noted that a revived current interest in the physiological significance of carbamate formation has arisen from their artifactual generation during the preparation of biofluids in bicarbonate buffers for multicomponent analysis by this technique [18,19].

2. MATERIALS AND METHODS

2.1 *Sample preparation and treatment*

Substance P was obtained from Cambridge Research Biochemicals (ICI, UK) and the dipeptide, Arg–Gly, was purchased from Sigma Chemical Co. (UK). 99.9% (v/v) ^2H_2O was obtained from Goss Scientific Ltd. (UK). Solutions of substance P (3.71×10^{-3} mol·dm^{-3}) and Arg–Gly (2.50×10^{-2} mol·dm^{-3}) in ^2H_2O were adjusted to pH 7.00 (meter reading) with ^2HCl and NaO^2H solutions in ^2H_2O prior to ^1H NMR analysis. Subsequently these solutions were treated with 2.50×10^{-2} mol·dm^{-3} $NaHCO_3$, and their pH values further adjusted to 7.00. ^1H NMR spectra of these HCO_3^--treated peptide solutions were acquired following equilibration at ambient temperature for periods of 30 min (substance P and Arg–Gly), 48 h (substance P) and 10 days (Arg–Gly).

2.2. *^1H NMR spectroscopy*

^1H NMR spectra were obtained from samples in 5 mm o.d. tubes using Bruker WH-400 and AMX-600 NMR spectrometers operating at 400 and 600 MHz, respectively. Spectra were acquired in the pulse-Fourier transform mode and are referenced to external trimethylsilyl-(2,2,3,3-$^2H_4$)-propionate (TSP). Typically up to 8,000 transients, each into 32k data points, were accumulated for each spectrum using a 45° flip angle pulse with a data acquisition time of ca. 3.5 s. Pre-irradation of the solvent residual ^2HOH signal for 2.5 s prior to each scan was employed to reduce the intensity of this resonance in the final spectrum. These long accumulation times were necessitated since the pre-irradiation was maintained at a low-power level in order to minimise the saturation of neighbouring α-CH resonances

3. RESULTS

Since the N-terminus of substance P is Arg–Pro–, the dipeptide, Arg–Gly, was primarily utilised as a simple, commercially available model compound for evaluating substance P–CO_2 interactions. The use of dipeptides as models for more complex peptides and proteins has much precedence in view of previous investigations by Roughton and Rossi-Bernardi [20] in which glycylglycine was employed to model carbamate formation in haemoglobin. The evaluation of model dipeptide carbamate production has particular advantages which are especially relevant to high-field NMR analysis since spectra are greatly simplified and CO_2-mediated modifications therein are readily detectable.

The 400 MHz ^1H NMR spectrum of a 2.50×10^{-2} mol·dm^{-3} solution of Arg–Gly in ^2H_2O (Fig. 1a) includes a triplet resonance located at 3.98 ppm which is assignable to the α-proton of the arginyl component, and full assignments for this spectrum are given in Table I. The p*K*a value of the arginyl α-amino group is likely to be close to 8.2 (cf. Arg–Ala[21]), giving rise to ca. 10–20% deprotonation of this primary α-amine function at physiological pH values. Subsequent to the addition of aqueous $NaHCO_3$ (2.50×10^{-2} mol·dm^{-3}), a new resonance centred at 4.08 ppm is generated in the spectrum (Fig. 1b). Although incompletely resolved, this signal appears to be a multiplet which, by analogy with previous investigations [22–24] is assignable to the α-proton of this dipeptide's carbamate adduct. This resonance appeared within 30 min of HCO_3^- addition, and was detectable in samples which were > 7 days old. Carbamate formation in this dipeptide also gave rise to a downfield shift in the position of the α-CH proton triplet. The terminal side-chain primary amine functional group of arginine is known to be quite resistant to carbamate production at neutral pH values [13] (p*K*a value ca. 10).

The generation of substance P carbamate in aqueous solution was also investigated by high-field NMR spectroscopy. A previous ^1H NMR study of substance P has shown that the peptide is largely aggregated in aqueous solution at extremes of pH (i.e. < 2 and > 9) [25] but that there is little or no aggregation at around pH 7.0, the pH used in this study. Chassaing et al. [26] used ^1H NMR in their study of the solvent dependence of substance P conformations; in particular they assigned the ^1H resonances in ^2H_2O solution at pD = 2.5. Our samples were at pH 7.00, and therefore the ^1H spectrum was re-investigated to check for a significant change in the ^1H chemical shifts between these two pH values. This re-assignment used COSY and TOCSY homonuclear two-dimensional ^1H spectra (described, for example, by Gray [27]) measured at 600 MHz. The ^1H chemical

Table I

^1H NMR data for Arg–Gly and Arg–Gly carbamate

δ	Assignment	Compound
1.70 (m)	Arg γ-CH_2	Arg-Gly
1.92 (m)	Arg β-CH_2	
3.26 (t)	Arg δ-CH_2	
3.98 (t)	Arg α-CH	
3.80 (d)	Gly α-CH	
3.85 (d)	Gly-α-CH′	
4.08 (m)	Arg α-CH	Arg-Gly carbamate

The sample consisted of a 2.5×10^{-2} mol·dm^{-3} solution of Arg–Gly in ^2H_2O at pH 7.0. The carbamate was formed by the addition of 2.5×10^{-2} mol·dm^{-3} $NaHCO_3$. The chemical shifts (δ) are in ppm with reference to high frequency external trimethylsilyl-(2,2,3,3-$^2H_4$)-propionate (TSP) The letters in parentheses indicate the multiplicity of the resonances: d, doublet; t, triplet; m, multiplet.

shifts measured in this study parallel those reported by Chassaing et al. [26] except that our shifts consistently have ca. 0.1 ppm higher frequency, a result of the difference in the external chemical shift reference substance (TSP here, tetramethylsilane in [26]). The only significant difference in relative chemical shifts is that, for the higher pH used here, the Arg^1 α-CH resonance has ca. 0.07 ppm lower frequency and overlaps with α-CH resonances from Gln^5 and Gln^6.

The partial ^1H spectrum of a 3.71×10^{-3} mol·dm^{-3} solution of substance P in ^2H_2O (pH 7.00) with corresponding resonance assignments is shown in Fig. 2a. Equilibration of this substance P solution with 2.50×10^{-2} mol·dm^{-3} HCO_3^- for a 30 min period (final pH 7.00) generated a new, relatively broad resonance at 4.13 ppm in the spectrum, a chemical shift value which is very close to that of the α-proton signal of amino acid carbamates [22]. Further HCO_3^--mediated modifications in the spectrum were also observed, including a downfield shift of ca. 0.1 ppm for the multiplets centred at 4.24 and 4.42 ppm in the spectrum of the untreated neuropeptide. Subsequent to further equilibration at ambient temperature (48 h), the carbamate signal appeared as a partially resolved multiplet located at 4.04 ppm. The chemical shift value of this carbamate resonance is virtually identical to that of the Arg–Gly dipeptide, and provides further evidence for carbamate generation in substance P under these experimental conditions.

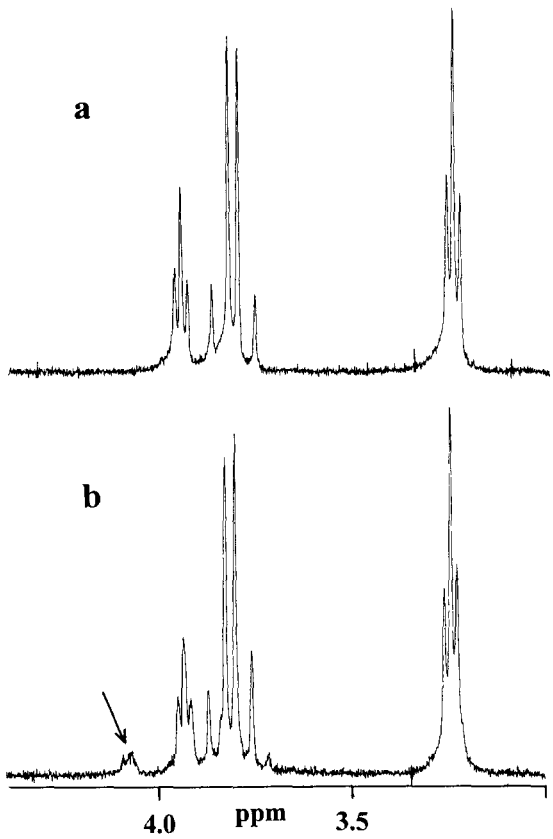

Fig. 1. Partial 400 MHz ^1H NMR spectra of a 2.50×10^{-2} mol dm^{-3} solution of the Arg–Gly dipeptide in ^2H_2O at pH 7.00 (a) prior and (b) subsequent to equilibration with 2.50×10^{-2} mol·dm^{-3} HCO_3^- at ambient temperature for 30 min. The arrow in spectrum b indicates the α-proton resonance of the dipeptide's carbamate adduct.

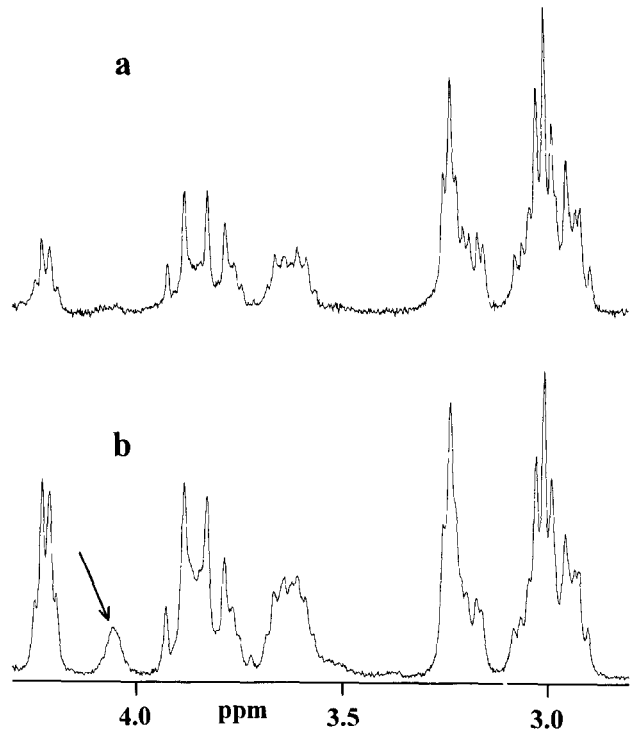

Fig. 2. Partial 400 MHz ^1H NMR spectra of a 3.71×10^{-3} mol·dm^{-3} solution of substance P in ^2H_2O at pH 7.00 (a) before and (b) after equilibration with 2.50×10^{-2} mol·dm^{-3} HCO_3^- at ambient temperature of a period of 30 min. The arrow in spectrum b indicates the α-proton resonance of the peptide's carbamate species.

The likely significance of substance P carbamate formation under physiological conditions can be judged from Fig. 3, in which the specification of a peptide at biologically relevant concentrations of bicarbonate has been modelled. The model uses a value of 8.00 for the p*K*a of the terminal amine functional group. The interaction of CO_2 with amine functional groups is usually defined in terms of an equilibrium constant K_c expressed in Eq. 2. In this equation the p*K*c value of the deprotonated amine function has been set to a value of 4.5.

$$K_c = \frac{[RNHCO_2^-]\,[H^+]}{[RNH_2]\,[CO_2]} \qquad (2)$$

4. DISCUSSION

Neurogenic inflammatory responses involving substance P have undoubtedly evolved as a protective mechanism and are likely to be involved in tissue repair. Under normal conditions, substance P may serve to

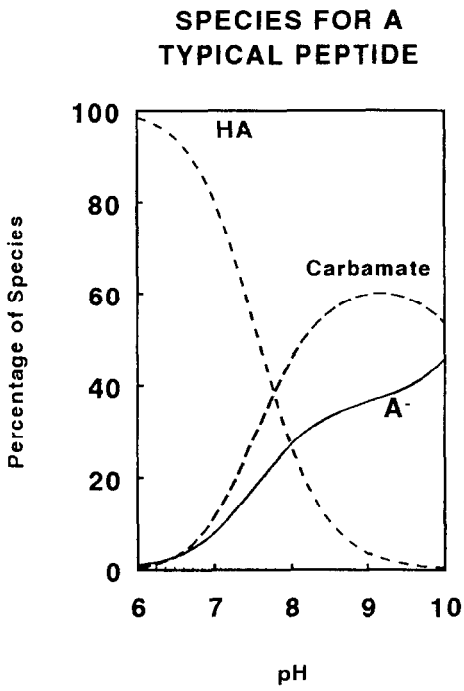

Fig. 3. Graphical representation of the extent of carbamate production for a typical peptide expressed as a function of physiological relevant HCO_3^- concentrations.

initiate or modulate the inflammatory response to noxious stimuli, ans diminishing levels of this neuropeptide may have deleterious effects on regulation of the inflammatory cascade. Indeed, it is conceivable that some aspects of the immune dysregulation seen in chronic arthritis arise from the depletion of neuropeptides such as substance P.

The High-field ^1H NMR data presented here provide much evidence for the reactions of the terminal α-amine functional groups of both substance P and the model dipeptide, Arg-Gly, with physiologically relevant concentrations of bicarbonate to form α-carbamate species. Since the estimated p*K*a value of the arginyl α-amino function in the parent peptide is ca. 8.2, the generation of carbamate species from substance P and Arg–Gly is not unexpected at physiologically relevant pH values.

The production of substance P carbamates in the hypercapnic environment of the inflamed rheumatoid joint is likely to be of much significance in view of previous reports that the diamino acids, α,γ-diaminobutyrate and α,β-diaminopropionate, exhibit neurotoxic behaviour only in the presence of bicarbonate [28]. Moreover, the neurotoxicity of L-cysteine, both in vivo and in vitro, is bicarbonate-dependent and mediated by *N*-methyl aspartate receptors [29]. Formation of the corresponding carbamates of these amino acids and peptides provides a plausible explanation for such activity. Indeed, L-cysteine readily forms an α-carbamate species, a phenomenon demonstrable by ^1H and ^{13}C NMR spectroscopy [24,30].

The generation of substance P α-carbamate in vivo may consequently exert an important influence on the vasoactivity of this neuropeptide which appears to play a critical role in inflammatory joint diseases, and we are currently investigating the ability of increasing concentrations of bicarbonate to influence its binding to cell surface receptors.

Acknowledgements: We wish to thank the Arthritis and Rheumatism Council (UK) for financial support, and the University of London Intercollegiate Research Service (ULIRS) for the ^1H NMR spectra acquired using the WH-400 and AMX-600 NMR spectrometers at Queen Mary and Westfield College (University of London).

REFERENCES

[1] Pernow, B. (1983) Pharmacol. Rev. 35, 85–141.
[2] Holzer, P. (1988) Neuroscience 24, 739–768.
[3] Foreman, J.C. (1987) Br. Med. Bull. 43, 386–400.
[4] Kidd, B.L., Mapp, P.I., Blake, D.R., Gibson, S.J. and Polak, J.M. (1990) Ann. Rheum. Dis 49, 644–652.
[5] Lund-Olsen, K. (1970) Arthritis Rheum. 13, 769–776.
[6] Treuhaft, P.S. and McCarty, D.J. (1971) Arthritis Rheum. 14, 475–484.
[7] Falchuck, K.H., Goetzl, E.J. and Kulka, J.P. (1970) Am. J. Med. 49, 22–33.
[8] Goetzl, E.J., Falchuck, K.H., Zergler, L.S. et al. (1971) J. Clin. Invest. 50, 1167–1180.
[9] Geborek, P., Saxne, T., Pettersson, H. and Wellheim, F.A. (1989) J. Rheumatol. 16, 468–472.
[10] James, M.J., Cleland, L.G., Rofe, A.M. and Leslie, A.L. (1990) J. Rheumatol. 17, 521–527.
[11] Naughton, D.P., Whelan, M., Smith, E.C., Williams, R., Blake, D.R. and Grootveld, M. (1993) FEBS Lett. 317, 135–138.
[12] Faurholt, C. (1925) J. Chim. Phys. 22, 1–44.
[13] Roughton, F.J.W. (1970) Biochem. J. 117, 801–812.
[14] Carbon Dioxide. Chemical, Biochemical and Physiological Aspects, US Government Printing Office (NASA no-5P-188), Washington.
[15] White, M.A., Handler, P. and Smith, E.L. (1986) Principles of Biochemistry, pp. 766–769, McGraw-Hill, 4th edn.
[16] Kilmartin, J.V. and Rossi-Bernardi, L. (1969) Nature 222, 1243.
[17] Dettman, H.D., Weiner, J.H. and Sykes, B.D. (1985) Can. J. Biochem. 63, 1120–1126.
[18] Sherry, D., Malloy, C.R., Jeffrey, F.M.H., Chavez, F. and Srere, H.K. (1990) J. Magn. Reson. 89, 391–395
[19] Connor, S.C., Ph.D. Thesis, University of London, (1989).
[20] Roughton, F.J.W. and Rossi-Bernardi, L. (1966) Proc. Roy. Soc. 164B, 381–400.
[21] Wojcik, J., Karczynski, F. and Kupryszewski, G. (1974) Rocz. Chem. 48, 1179–1186.
[22] Nunn, P.B. and O'Brien, P. (1989) FEBS Lett. 251, 31–35
[23] Davis, A.J., Hawkes, G.E., O'Brien, P., Wang, G. and Nunn, P.B. (1991) J. Chem Res. (5), 84–85.
[24] Morrow, J.S., Keim, P. and Gurd, F.N. (1974) J. Biol. Chem. 249, 7484–7494.
[25] Poujade, C., Lavielle, S., Chassaing, G. and Marquet, A. (1983) Biochem. Biophys. Res. Commun. 114, 1109–1116.
[26] Chassaing, G., Convert, O. and Lavielle, S. (1986) Eur. J. Biochem. 154, 77–85.
[27] Gray, G.A. (1988) in Pulse Methods in 1D and 2D Liquid-phase NMR (W.S. Brey ed.) Academic Press, ch. 5.
[28] Weiss, J.H., Christine, C.W. and Choi, D.W. (1989) Neuron 3, 321.
[29] Olney, J.W., Zorumski, C., Price, M.T. and Labruyer, J. (1990) Science 248, 596–599.
[30] Nunn, P.B., Davis, A.J. and O'Brien, P. (1991) Science 251, 1619.