Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 108-113

COMPETITIVE PEPTIDE ANTAGONISTS OF ANF-INDUCED CYCLIC GUANOSINE MONOPHOSPHATE PRODUCTION

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Received August 16, 1989

Atrial natriuretic factor (isoleucine ANF 101-126), cleaved ANF (isoleucine ANF 101-105/106-126) and des (Gln 18, Ser 19, Gly 20, Leu 21, Gly 22) ANF 4-23-NH2 (C-ANF 4-23) stimulated cyclic guanosine monophosphate production (cGMP) by rat aortic vascular smooth muscle cells (VSMC) in culture. Cleaved ANF and ANF C4-23 also antagonised or diminished the response to ANF 101-126. Agonist and antagonist actions of both peptides were dose-related. In contrast, prepro ANF (104-123), an ANF precursor fragment, exhibited no agonist or antagonist effect on cGMP production.

Atrial natriuretic peptide continues to excite attention as a circulating peptide hormone of potentially major importance in pressure/volume homeostasis. Information concerning degradation of ANF in vivo is scant. We have recently demonstrated the presence of a ring cleaved form of ANF (ANF 99-105/106-126) in human plasma (1) which appears to be the initial ANF degradation metabolite generated by neutral metallo-endopeptidase (EC 3.4.24.11) (2). This ring-cleaved form of ANF constitutes up to 30% of ANF immunoreactivity detected in human coronary sinus plasma (1). There is little known concerning the bioactivity of this ANF metabolite or its potential to interact with, and possibly modulate the action of, intact ANF. We investigated the effect of cleaved ANF on cGMP production by cultured rat vascular smooth muscle cells (VSMC) and the interaction of cleaved with intact ANF with respect to production of this nucleotide intracellular second messenger. The effects of cleaved ANF were compared with those of c-ANF 4-23, a ring-diminished analogue of ANF previously thought to be devoid of in vitro bioactivity (3), and with prepro ANF (104-123), an ANF precursor fragment which has reportedly induced cGMP production in kidney tissue homogenates (4).

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0006-291X/89 $1.50

METHODS

Peptides

Isoleucine ANF 101-126 was supplied by Merck, Sharpe and Dohme, Rahway, New Jersey. Cleaved rat ANF (ANF 101-105/106-126) was made by incubation of synthetic rat ANF (101-126) with thermolysin (Sigma Chemicals, St Louis, MD) and purified by HPLC. Amino acid analysis of the cleaved ANF preparation yielded mole ratios consistent with intact ANF (101-126) but sequence analysis identified two free N termini beginning at Arg 101 and phe 106 proving the product to be ANF (101-126) cleaved between residues cys 105 and phe 106. No intact rat ANF (101-126) or other major products could be detected when 30ug of cleaved ANF was analysed by HPLC which indicated less than <0.3% of other peptides. Amino acid analysis yielded 72% peptide by weight. des [Gln 18, Ser 19, Gly 20, Leu 21, Gly 22] ANF 4-23-NH (C-ANF 4-23), and human Prepro-ANF 104-123 were obtained from Peninsula Laboratories (Belmont, CA).

Cell Cultures

Cultures of vascular smooth muscle cells (VSMC) were prepared by enzymatic dispersion from the aortae of 12 week old Wistar-Kyoto rats (as previously described (5). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Gibco) and antibiotics (penicillin 100u/ml, streptomycin 100ugm/ml and amphotericin B 0.25ugm/ml). The VSMC were serially subcultured and used from passages 4-16.

Experiment

VSMC were grown to confluence in 12-well plates (4.5cm^2, Libro). Wells were seeded with 1-2 x 10^5 VSMC. Two to four days after plating, confluent cell monolayers were washed once with DMEM supplemented with 2% FCS and incubated with or without 5 x 10^{-8}M isoleucine ANF 101-126. Peptides (cleaved ANF, C-ANF 4-23 and prepro ANF) were added at concentrations of 0, 5 x 10^{-8}, 5 x 10^{-7} and 5 x 10^{-6}M. The phosphodiesterase inhibitor, 3-Isobutyl-1-methyl-xanthime, 0.25mM (IBMX, Aldrich Chemical Co., Milw., W.I.) was added to all cultures. Culture supernatants were collected after 30 minutes incubation at 37°C in 5% CO_2. Extracellular cGMP concentrations were determined by direct, non-acetylated radioimmunoassay (6). Data were expressed as mean percentages ± SEM of cGMP produced relative to the positive control (i.e. effect of 5 x 10^{-8}M ANF alone). Statistical comparisons were performed by two factor analysis of variance with repeated measures. Comparisons of means of absolute values (pmol cGMP/ml) were conducted using Fisher's protected least significant difference test.

RESULTS

Intact isoleucine ANF 101-126 (5 X 10^{-8}M) induced elevation of extracellular levels of cGMP in cultures of rat VSMC to 24-fold control values (12.6 ± 2.1 vs 0.5 ± 0.06pmol/ml, n=18, p<0.001). Cleaved ANF alone stimulated cGMP production in a dose-dependent fashion (Fig.1) yielding cGMP concentrations of 0.77, 1.38, 2.53, 2.70 and 3.05pmol/ml in response to cleaved ANF concentrations of 0, 5 x 10^{-8}M, 5 x 10^{-7}M, 5 x 10^{-6}M and 10^{-5}M respectively ($p<0.05$ for 5 x 10^{-8}M and $p<0.001$ for all higher doses of cleaved ANF in comparison with administration of nil peptide). When administered together with intact ANF (5 x 10^{-8}M), cleaved ANF exhibited antagonist activity diminishing the cGMP responses to intact ANF at ratios of 200:1 (10^{-5}M cleaved

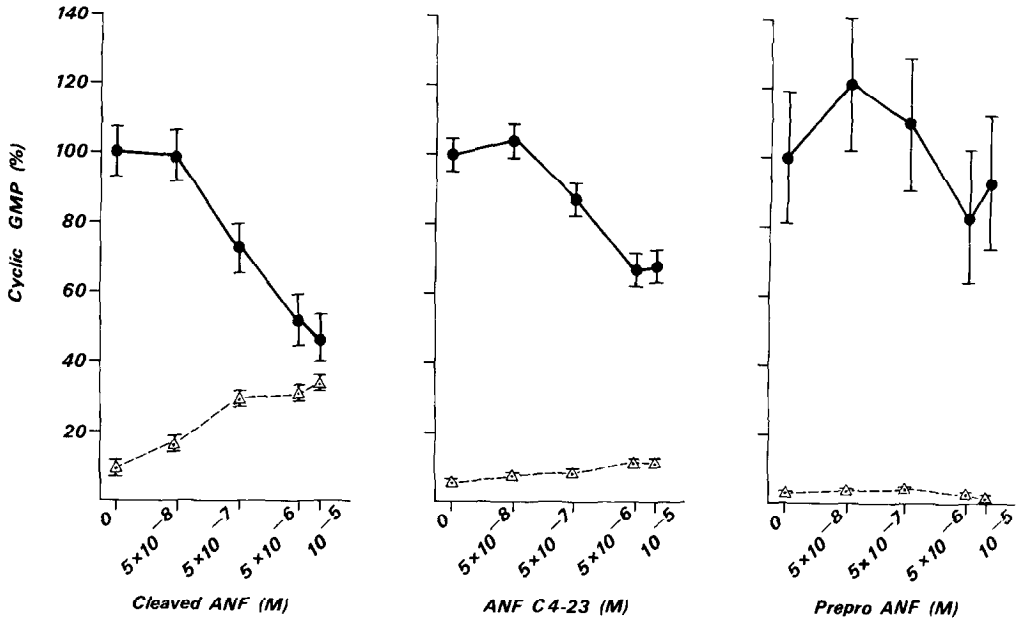

Figure 1.
Extracellular cyclic GMP levels in cultured VSMC (mean ± SEM, expressed as percentage of concentrations induced in presence of 5 x 10^{-8}M intact ANF 101-126 alone) stimulated by increasing concentrations of cleaved ANF (left), cANF 4-23 (centre) and prepro ANF (ANF 79-98) (right) administered alone (△) and against a constant background dose (5 x 10^{-8}M) of ANF 101-126 (●). Statistical analysis was conducted using absolute levels of cGMP production. All doses of cleaved ANF exhibited significant agonist activity and doses of 5 x 10^{-7}M or greater were significantly antagonistic. C-ANF 4-23 exhibited significant agonist and antagonist activity at concentrations of 5 x 10^{-7}M or greater (see RESULTS text for absolute data and statistical significance). Prepro ANF (ANF 79-98) exhibited neither agonist nor antagonist activity.

ANF: 5 x 10^{-8} ANF 101-126) by 53% and by 48% at 100:1 ($p<0.001$ for both). At the lower concentration of 5 x 10^{-7}M cleaved ANF (10:1), 28% inhibition was observed (Fig.1, $p<0.02$).

The ring-diminished ANF analogue c-ANF 4-23 also stimulated cGMP in a concentration-dependent fashion (Fig.1) up to 2-fold control values when administered at peak concentrations of 5 x 10^{-6} and 10^{-5}M ($p<0.001$). C-ANF 4-23 exhibited specific antagonism of cGMP production induced by ANF 101-126 (Fig.1).

In contrast to cleaved ANF and C-ANF 4-23, prepro ANF 104-123 neither stimulated cGMP production when administered alone nor antagonised the action of intact ANF 101-126 (Fig.1).

DISCUSSION

Our data indicate that the ANF metabolite, ANF 101-105/106-126 ("cleaved ANF") induces cGMP production from cultured VSMC in a dose-dependent fashion. At 5

x 10^{-8}M cleaved ANF induced cGMP levels a little less than one-fifth those stimulated by a similar concentration of intact ANF 101-126 (Fig.1). Interestingly, the cleaved product exhibited potent dose-related antagonism of cGMP production induced by intact ANF. At micromolar concentrations cleaved ANF virtually eliminated the additional cGMP concentrations promoted by 5 x 10^{-8}M ANF 101-126 with the residual level of cGMP observed being no more than that attributable to the agonist effect of cleaved ANF alone (Fig.1). Cleaved ANF may constitute up to 30% of ANF immunoreactivity detectable in human coronary sinus plasma (1). This proportion falls well short of the 10:1 ratio at which antagonism was first observed in the current experiments. However, the possibility still remains that this metabolite of ANF may modulate the effects of the intact peptide possibly at, or near, the sites of its production by neutral metalloendopeptidase where tissue levels of cleaved ANF are conceivably very high.

To date investigators have not had access to a specific peptide antagonist of ANF for use as a probe to assist in defining the role of ANF in normal physiology or in the pathophysiology of such conditions as hypertension and heart failure. Our data suggest that cleaved ANF may partially fulfill such a role. It is certainly not a pure antagonist of intact ANF as it possesses weak agonist activity of its own, but it is possible it may act as an effective antagonist when the ANF system is markedly activated in a fashion analogous to the actions of the partial angiotensin II antagonist, saralasin, in the presence of enhanced activity of the renin-angiotensin system. Cleaved ANF may thus be a useful probe for exploration of the role of ANF in experimental models of hypertension and heart failure.

Our choice of C-ANF 4-23 for comparison with the effects of cleaved ANF was intended to provide negative data to contrast with data derived from studies of cleaved ANF. To our surprise this ring-diminished ANF analogue, repeatedly declared to be devoid of in vitro bioactivity (3), exhibited a qualitatively similar, though distinctly weaker, pattern of dose-related agonist and antagonist actions as seen with cleaved ANF (Fig.1). The agonist and antagonist effects were first apparent at concentrations of 5 x 10^{-8} and 5 x 10^{-7}M respectively (Fig.1). C-ANF 4-23 has been used to support the concept of biologically inactive "clearance" binding sites for ANF which allegedly constitute over 90% of ANF receptors (3). This ANF analogue has been reported as being biologically inert in vitro but able to promote an increase in plasma ANF concentrations and natriuresis in vivo. These findings are thought to reflect displacement of endogenous ANF from clearance receptors making more ANF available to guanylate cyclase linked receptors and thus producing in vivo bioactivity. At the doses used in those previous studies (3) C-ANF 4-23 would

be essentially inactive in vitro as judged by our current data. However, our findings indicate that the lack of in vitro bioactivity of C-ANF 4-23 is relative rather than absolute. Hence, investigators should be alerted that at very high concentrations, this ANF analogue may exert agonist and/or competitive antagonist effects which may potentially confound interpretation of experimental results.

In contrast to a previous report (4) we were unable to detect any agonist activity of the ANF preprohormone N-terminal fragment "ANF (104-123)". Nor did this peptide exhibit antagonism to intact ANF. This peptide forms a portion of prepro ANF and in current nomenclature would be better designated "ANF 79-98". Hence it does not contain the biologically active carboxy terminal portion of the ANF prohormone with the ring structure generally accepted as necessary for bioactivity. Hence we find its lack of biological effect in the current studies unsurprising. Experimental circumstances differed between our studies and those of Vesely et al (4) in that we investigated effects on cultured VSMC rather than preparations of renal cortical membranes or whole kidney homogenates. It is conceivable that cGMP responses are tissue specific for any given putative agonist peptide and this may partially explain the discrepancy between our findings and the results reported previously (4).

In summary, cleaved ANF, an endogenous metabolite of ANF, showed weak agonist and significant antagonist effects as evidenced by its effect on cGMP production by cultured VSMC. This peptide may provide a useful probe to aid definition of the role of ANF in pressure/volume homeostasis in pathophysiological states. C-ANF 4-23, previously regarded as inert in vitro, exhibited a similar though weaker pattern of agonist and antagonist activity and such effects must be considered by investigators undertaking experiments employing high doses of this ANF analogue. A prepro ANF fragment (ANF 79-98), previously reported to induce cGMP production from preparations of renal tissue, did not induce detectable changes in cGMP production from cultured VSMC.

ACKNOWLEDGMENTS

Support was provided by the Medical Research Council and the National Heart Foundation of New Zealand. Steve Brennan and Maurice Owen (Department of Pathology, Christchurch Hospital) performed amino acid sequencing and analysis to characterise cleaved ANF.

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