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Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle

(vasodilation/nitrovasodilators/cyclic GMP)

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ABSTRACT **A 26 amino acid synthetic peptide fragment of atrial natriuretic factor (ANF) relaxed isolated rabbit aortic segments in which the endothelium was either intact or functionally destroyed. The relaxations were temporally associated with increases in levels of cGMP with no change in the levels of cAMP. The ANF-induced increases in cGMP were also observed in aortic segments pretreated with calcium-free buffer or the cGMP phosphodiesterase inhibitor M&B 22,948. Qualitatively similar results were obtained for sodium nitroprusside. ANF selectively activated particulate guanylate cyclase, having no effect on the soluble form of the enzyme. Thus, the direct (endothelium-independent) vasodilator effect of ANF may be mediated via increased tissue levels of cGMP. ANF appears to increase vascular cGMP levels by activation of particulate guanylate cyclase.**

Mammalian atria contain several peptides that may play an important role in regulating electrolyte balance and vascular resistance (1, 2). The elucidation of the amino acid sequence of these bioactive peptides (3–6) has led to the production of synthetic analogs (5), which will allow a more precise characterization of the pharmacology of atrial natriuretic factor (ANF). Recently, using one such synthetic peptide (26 amino acid fragment), the vasodilator profile of ANF was assessed and found to be qualitatively similar to the profile for sodium nitroprusside but not for other commonly used vasodilators, such as adenosine, papaverine, or nifedipine (7). The nitrovasodilators are thought to induce smooth muscle relaxation through activation of guanylate cyclase leading to the formation of cGMP (8–10). Furthermore, the relaxation and increase in the levels of cGMP in vascular smooth muscle produced by nitrovasodilators are independent of the vascular endothelium, in contrast to similar changes produced by endothelium-dependent vasodilators, such as acetylcholine (11–14). Thus, the ability of ANF to activate guanylate cyclase and to alter the levels of cGMP and cAMP was investigated and compared to the actions of the nitrovasodilator sodium nitroprusside.

METHODS

Male New Zealand White rabbits (2–2.5 kg) were stunned by a blow to the head and exsanguinated. The thoracic aorta was removed and quickly placed in physiological salt solution of the following composition: 130 mM NaCl/4.7 mM KCl/1.18 mM KH_2PO_4/1.17 mM $MgSO_4$/1.6 mM $CaCl_2$/14.9 mM $NaHCO_3$/11.0 mM dextrose/0.026 mM EDTA, equilibrated with 95% O_2/5% CO_2 (pH 7.2). Vessels were cleaned of extraneous tissue under a dissecting microscope taking care not to damage the lumenal surface. However, in a few aortas, the endothelium was functionally destroyed by rubbing the lumen with a wooden stick (15). Ring segments (4–5 mm long) were cut from each aorta and mounted in glass tissue baths (50 ml) for monitoring changes in isometric force. Aortic rings were stretched to a resting force (2.0 g), which allowed for optimal levels of contractile force to be generated. Tissues were washed with fresh oxygenated physiological salt solution (38°C) every 20 min. Following a 1-hr equilibration period, rings were contracted by exogenous serotonin (0.3 μM, an approximate EC_{80} concentration). Either ANF (10 nM) or sodium nitroprusside (1 μM) was added once the contractile response had reached an equilibrated plateau. In the experiments involving calcium-free buffer (no added calcium, 1 mM EGTA) or 0.1 mM M&B 22,948, the particular treatment was initiated 10 min prior to the addition of serotonin.

cGMP and cAMP levels were assayed as described (8) in aortic ring segments (10 mm long) that had been rapidly frozen in liquid nitrogen. Rings were suspended without tension in tissue baths and then subjected to serotonin (0.3 μM) for 20 min prior to the addition of either ANF (10 nM) or sodium nitroprusside (1 μM). Tissues were frozen at the designated time points and homogenized in 6% trichloroacetic acid; the extracts were kept at −70°C until assayed. Samples were thawed and centrifuged (low speed for 60 min), the supernatants were extracted with ether, and a portion of the extract was acetylated (16) and radioimmunoassayed for cyclic nucleotides (17).

Guanylate cyclase was assayed as described (18). Aortas were homogenized in 50 mM Tris·HCl (pH 8.0) containing 1 mM EDTA/250 mM sucrose/1 mM dithiothreitol. Homogenates were centrifuged at 105,000 × g for 60 min and the pellets were resuspended in homogenization buffer. Guanylate cyclase was assayed in 10 μl of supernatant (10 μg of protein) or particulate (100 μg of protein) fractions at 37°C in the presence of 50 mM Tris·HCl, pH 7.6/10 mM theophylline/2 mM isobutylmethylxanthine/2 mM M&B 22,948/15 mM creatine phosphate/20 μg of creatine phosphokinase (135 units/kg) in a final volume of 0.1 ml. Reactions were initiated by the addition of substrate (4 mM $MgCl_2$/1 mM GTP) and terminated with 0.9 ml of 50 mM sodium acetate (pH 4.0) followed by immersion for 3 min in a boiling water bath. cGMP formed was quantified by radioimmunoassay. Data are expressed per mg of protein. Significance was accepted at the 0.05 level of probability using Student's t test.

Drugs used were synthetic ANF [26 amino acid fragment, 8–33 COOH-terminal acid (5), Department of Medicinal

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Abbreviation: ANF, synthetic atrial natriurectic factor (H-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH).

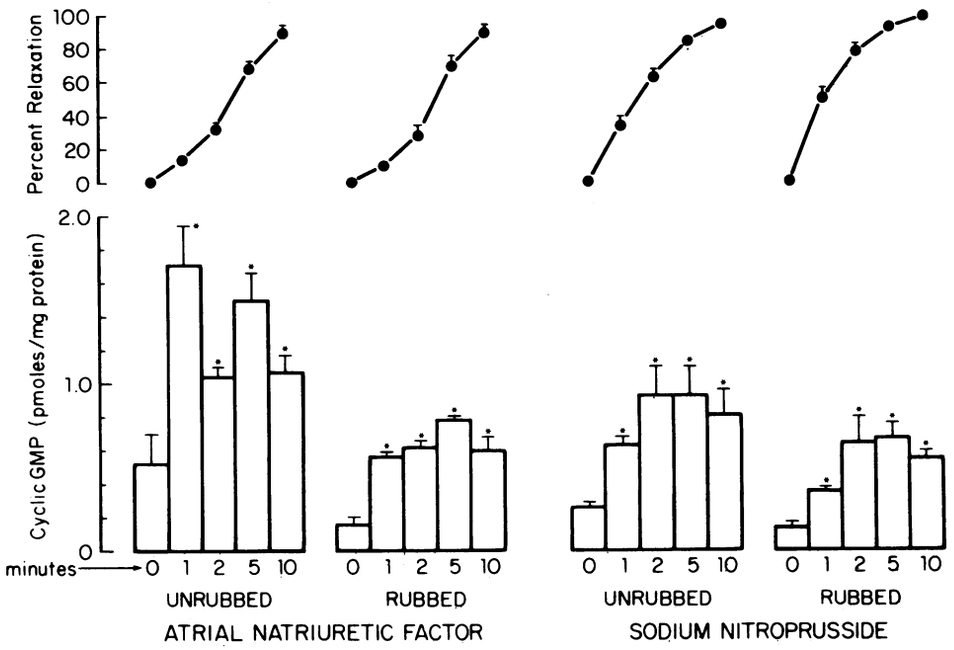

FIG. 1. Time course for relaxation and changes in cGMP in rubbed and unrubbed rabbit aortic segments after addition of either ANF (10 nM) or sodium nitroprusside (1 μM). Data are shown as mean values ± SEM for percentage relaxation ($n = 4$) and tissue levels of cGMP ($n = 3$–4, pmol per mg of protein). Asterisk denotes a significant increase in cGMP over the zero time (pre-ANF) value.

Chemistry, Merck], sodium nitroprusside (Nipride, Roche), serotonin, and acetylcholine (Sigma). M&B 22,948 was a gift from May and Baker (Dagenham, Essex, U.K.). Other materials were obtained as described (8, 18).

RESULTS

Exogenous ANF (10 nM) and sodium nitroprusside (1 μM) caused complete relaxations both in intact aortic rings and in rings in which the endothelial surface had been functionally destroyed by rubbing with a wooden stick (Fig. 1). Previous work had determined that these concentrations were approximately equally effective in relaxing serotonin-contracted aortic rings (7). Neither acetylcholine (1 nM to 1 μM), substance P (0.1 nM to 0.1 μM), nor cholecystokinin (1 nM to 0.1 μM) relaxed the rubbed aortic preparations (data not shown). The same concentrations of ANF and sodium nitroprusside that induced relaxation also caused significant increases in the levels of cGMP in aortic segments (Fig. 1). The increases in cGMP were evident in both rubbed and unrubbed aortic segments. The increases in cGMP with ANF were also observed in the absence of contraction with serotonin (data not shown). ANF appeared to cause a greater increase in cGMP in the unrubbed versus the rubbed tissues. However, basal values of cGMP (pmol/mg of protein) were higher in the unrubbed (518 ± 181) compared to rubbed (162 ± 40) tissues, so that the percentage increases of cGMP in response to ANF were comparable. ANF failed to elicit any change in the levels of cAMP in these tissues (data not shown).

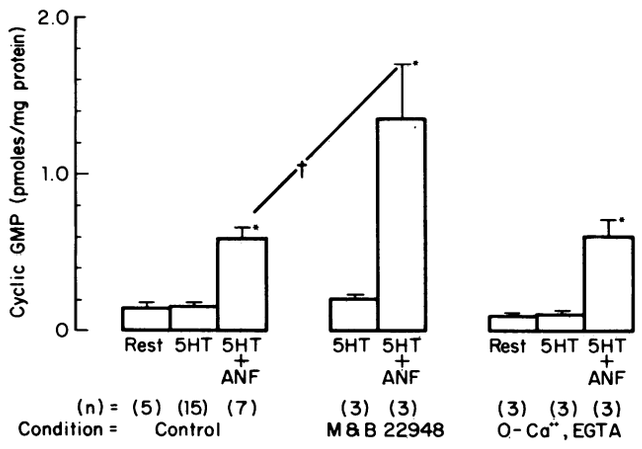

FIG. 2. Effects of ANF (10 nM) on levels of cGMP in control tissues and in tissues pretreated with either M&B 22,948 (0.1 mM) or calcium-free buffer (0-Ca^{++}, EGTA). Serotonin (5HT, 0.3 μM) was added prior to addition of ANF. All of the aortic segments had their lumen surface rubbed with a wooden stick. Numbers in parenthesis refer to number of tissues (and therefore animals) used for each determination. Values are means ± SEM. * and † denote significance at the $P < 0.05$ level, with the asterisk referring to a significant increase in cGMP over the 5HT-treated tissues.

Additional experiments were performed to elucidate the mechanisms underlying the ANF-induced increase in cGMP. ANF caused a significant increase in cGMP in aortic segments that had been pretreated with the phosphodiesterase inhibitor M&B 22,948 (0.1 mM) (Fig. 2). The ANF-induced increase in cGMP in the presence of M&B 22,948 was significantly enhanced compared to untreated tissues. ANF also increased cGMP in aortic segments that had been incubated in calcium-free physiological saline solution (Fig. 2). Basal levels of cGMP were lowered by removal of calcium from the buffer. We also examined the effects of ANF on particulate and soluble guanylate cyclase isolated from rabbit aorta. The addition of ANF (10 nM, 1 μM) caused a significant activation of particulate guanylate cyclase (Table 1). ANF

Table 1. Effects of ANF on particulate and soluble forms of guanylate cyclase isolated from rabbit aorta

		% basal activity after ANF	
	Basal activity* ($n = 8$)	10 nM ($n = 6$)	1 μM ($n = 8$)
Soluble	46.4 ± 5.3	101 ± 4	96 ± 4
Particulate	6.7 ± 0.6	149 ± 21†	157 ± 11†

*pmol of cGMP per mg of protein per min.
†$P < 0.05$.

(up to 1 μM) had no effect on the soluble form of guanylate cyclase.

DISCUSSION

A previous report (7) indicated that the vasodilator profile of ANF was similar to that found for sodium nitroprusside but not other commonly used vasodilators (i.e., papaverine, nifedipine, adenosine). Therefore, the present study was designed to examine possible mechanisms underlying the vasodilator activity of ANF in an attempt to determine whether ANF and the nitrovasodilators share a common mode of action in relaxation of vascular tissue. Elucidation of these mechanisms should be of predictive value in assessing therapeutic indications for synthetic analogs of ANF.

Exogenous ANF elicited a complete relaxation whether or not the structural integrity of the vascular endothelium was preserved (Fig. 1). These relaxations were coincident with significant increases in tissue levels of cGMP, while cAMP levels remained unaltered. We assume that the endothelium was functionally destroyed in our rubbed tissues, because the rubbed aortic rings, when contracted with serotonin, failed to relax to acetylcholine, substance P, or cholecystokinin (up to 1 μM), agents previously described as endothelium-dependent vasodilators (12, 15). Furthermore, the basal levels of cGMP were lower in the rubbed versus unrubbed aortic segments. Previous studies have demonstrated that the levels of cGMP are reduced in vascular tissue without an intact endothelium (11–14). Our results showing an endothelium-independent relaxation and increase in cGMP elicited by ANF have only been observed for the nitrovasodilator class of relaxants (refs. 12–14, 19; present study).

The ANF-induced increase in cGMP levels was potentiated in tissues pretreated with M&B 22,948 (0.1 mM; Fig. 2), a compound that has been shown to be a selective inhibitor of cGMP phosphodiesterase (20). The concentration used in our study (0.1 mM) is approximately 6 times the K_i value for inhibition of cGMP hydrolysis in bovine coronary artery (20). Thus, it appears unlikely that the accumulation of cGMP in response to ANF is mediated via inhibition of phosphodiesterase. Qualitatively similar results with ANF were obtained in the presence of isobutylmethylxanthine (10 μM), a relatively nonselective phosphodiesterase inhibitor (data not shown). The increase in cGMP following ANF was not dependent on the presence of extracellular calcium (Fig. 2). Increases in cGMP levels caused by hormones that directly act on the smooth muscle typically require the presence and, therefore, presumably the influx of extracellular calcium (11). In contrast, nitrovasodilators (11) and ANF increase cGMP independent of extracellular calcium. Functionally destroying the endothelium or incubation of aortic segments in the absence of extracellular calcium resulted in diminished basal levels of cGMP as reported previously (9, 11, 13, 19).

ANF appears to increase cGMP via activation of particulate but not soluble guanylate cyclase (Table 1). The degree of activation achieved by ANF is similar to that found for the heat-stable enterotoxin, produced by *Escherichia coli*, of intestinal particulate enzyme (21–24). Heat-stable toxin is one of the only other known select activators of particulate guanylate cyclase (21, 23).

The nitrovasodilators cause a predominant, if not exclusive, activation of the soluble form of guanylate cyclase (19). Indeed, a maximally effective concentration of sodium nitroprusside (100 μM) caused a 10-fold activation of guanylate cyclase obtained from rabbit aorta (data not shown). Little activation was observed with concentrations of sodium nitroprusside (1 μM) that stimulated maximum relaxation of rabbit aorta. However, others have reported that incorporation of various compounds, including porphyrins or reducing agents, into enzyme incubations results in a marked shift to the left in the dose–response curve to various nitrovasodilators (11). Therefore, it is difficult to mimic the exact conditions under which guanylate cyclase activation occurs in intact cells. Thus, the maximal activation of soluble guanylate cyclase with 100 μM sodium nitroprusside in our assay conditions is not inconsistent with a role for this enzyme in vascular smooth muscle relaxation.

Similar experiments studying the effects of sodium nitroprusside (100 μM) on particulate preparations from rabbit and rat aorta resulted in only slight activation of guanylate cyclase (ref. 19; unpublished observations). However, studies of the effects of nitrovasodilators on guanylate cyclase in particulate fractions remain equivocal, because these preparations may be contaminated as much as 50% with soluble guanylate cyclase (25, 26). Even with extensive washing of particulate fractions with high ionic strength buffers, contamination with soluble guanylate cyclase may be as great as 10% (25, 26). Therefore, it is difficult to ascribe small activations by nitrovasodilators in high-speed pellets to either the soluble or particulate form of guanylate cyclase.

Our results demonstrate that ANF and the nitrovasodilators share a number of similarities in their relaxant effects on vascular smooth muscle. Both compounds elicit an endothelium-independent vasodilation that is temporally correlated with an increased tissue level of cGMP. Whether these properties are unique to ANF or are shared by other peptides remains to be determined. Both ANF and the nitrovasodilators are further distinguished from other relaxants in their ability to increase cGMP independent of either phosphodiesterase inhibition or the presence of extracellular calcium. Finally, activation of guanylate cyclase is yet another similarity exhibited by these compounds. However, our finding that ANF selectively activates the particulate enzyme may be of value in determining whether activation of distinct populations of guanylate cyclase (i.e., particulate versus soluble) results in the expression of quantitatively or even qualitatively different physiological responses amongst the vascular tree. It is of interest to note that select activation of different populations of adenylate cyclase has been suggested to result in either inotropism or chronotropism in cardiac muscle (27). Of obvious importance is determining whether this mechanistic profile of ANF will lead to the development of a novel class of therapeutic agent for cardiovascular disorders.

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