The presence of atrial-natriuretic-factor receptors of ANF-R2 subtype in rat platelets

Coupling to adenylate cyclase/cyclic AMP signal-transduction system

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The effect of atrial natriuretic factor (ANF) on adenylate cyclase activity was studied in rat platelet membranes. ANF-(99–126)-, -(101–126)-, -(103–126)- and -(103–123)-peptide inhibited adenylate cyclase activity in a concentration-dependent manner with an order of potency of ANF-(103–123)-peptide > ANF-(99–126)-peptide > ANF-(101–126)-peptide > ANF-(103–126)-peptide. ANF-(103–123)-peptide and ANF-(99–126)-peptide inhibited the enzyme activity by about 50–55 %, with an apparent K_i between 0.1 and 0.5 nM, and ANF-(101–126)-peptide inhibited the enzyme activity by about 35 %, with an apparent K_i between 1 and 3 nM. On the other hand, ANF-(103–126)-peptide was the least potent and inhibited the adenylate cyclase activity by about 30 % ($K_i \sim 10$ nM). The inhibitory effect of ANF on adenylate cyclase was also dependent on the presence of guanine nucleotides and was attenuated by amiloride and pertussis toxin. The stimulatory effects of various agonists such as *N*-ethylcarboxamideadenosine, prostaglandin E_1 , isoprenaline and forskolin on adenylate cyclase were also inhibited by ANF to various extents; however, the stimulations were not completely abolished. In addition, ^{125}I -labelled ANF-(99–126)-peptide bound specifically to rat platelet membranes. The binding of ^{125}I -ANF was competitively inhibited in a concentration-dependent manner by the unlabelled peptides which were used for adenylate cyclase inhibition. ANF-(103–123)-peptide, ANF-(99–126)-peptide and ANF-(101–126)-peptide were almost equipotent [IC_{50} (median inhibitory concentration) = 0.1–1 nM], and ANF-(103–126)-peptide was the least potent ($\text{IC}_{50} \sim 10$ nM). Scatchard analysis of the data revealed the presence of a single class of binding sites of high affinity ($K_d \sim 120$ pM). Affinity cross-linking of ^{125}I -ANF-(99–126)-peptide to its binding sites in rat platelet membranes and analysis by SDS/PAGE followed by autoradiography showed a predominant labelling of a protein band with an apparent M_r of 66000. These data indicate the presence of only ANF-R2 (low- M_r) receptors in platelets and suggest that these receptors may be coupled to the adenylate cyclase system.

INTRODUCTION

Atrial natriuretic factor (ANF) from mammalian atria have been reported to exhibit a variety of physiological effects, including diuresis, natriuresis, vasorelaxation, inhibition of aldosterone secretion from adrenal cortex and inhibition of vasopressin and renin release [1]. ANF has been reported to stimulate guanylate cyclase/cyclic GMP [2,3] and to inhibit the adenylate cyclase/cyclic AMP system in several tissues [4–11], suggesting that these two second-messenger systems may be responsible in mediating the physiological responses of ANF. By using affinity cross-linking techniques [12] or photo-affinity labelling [13], at least two classes of ANF receptors have been shown to be present in several tissues [12–15]. These are of high (130000) and low (66000) M_r and have been designated 'ANF-R1' and 'ANF-R2' receptors respectively [12]. ANF-R1 receptors contain an intrinsic particulate guanylate cyclase [12], whereas ANF-R2 receptors have been shown to be non-guanylate-cyclase-coupled. Recently, Maack and his colleagues [16] have demonstrated that ANF-R2 receptors are biologically silent receptors (the primary function of these receptors is the sequestration and metabolic clearance of ANF) and they have therefore designated them 'clearance receptors' (C-ANF receptors). However, we have

shown recently that the C-ANF receptors are coupled to adenylate cyclase/cyclic AMP system [17].

ANF-receptor-binding sites have been shown to be present in human platelet membranes [18]. Since particulate guanylate cyclase is not found in platelets and ANF has not been shown to alter cyclic GMP levels in platelets [19], it is possible that the ANF receptors in platelets may be of ANF-R2 subtype and are coupled to the adenylate cyclase system. The present studies were undertaken to investigate this possibility.

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EXPERIMENTAL

Materials

ATP, cyclic AMP and isoprenaline were purchased from Sigma (St. Louis, MO, U.S.A.). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3) and guanosine 5'-[γ -thio]triphosphate (GTP[S]) were purchased from Boehringer-Mannheim, Montreal, Quebec, Canada. [α - ^{32}P]ATP was from Amersham, Oakville, Ontario, Canada, and *N*-ethylcarboxamideadenosine (NECA) was from Research Biochemicals, Wayland, MA, U.S.A. Rat

Abbreviations used: ANF, atrial natriuretic factor; GTP[S], guanosine 5'-[γ -thio]triphosphate; NECA, *N*-ethylcarboxamideadenosine; G_i , inhibitory guanine-nucleotide-regulatory protein; C-ANF_{4–23}, des-Gln¹⁸-Ser¹⁹-Gln²⁰-Leu²¹-Gly²²-ANF-(4–23)-peptide-NH₂; IC_{50} , median inhibitory concentration; PT, pertussis toxin.

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ANF-(99–126)-peptide, ANF-(101–126)-peptide and ANF-(103–123)-peptide and atriopeptin I were kindly provided by Bio-Méga, Laval, Quebec, Canada.

Methods

Preparation of platelet membranes. Rat platelet membranes were prepared with minor modifications of the method described by Schiffrin *et al.* [18]. Blood was drawn from femoral arteries of anaesthetized rats into citrate anticoagulant (87 mM-sodium citrate/105 mM-dextrose/11 mM-citric acid/7 mM- Na_3PO_4). A 1 ml portion of anticoagulant was used for each 10 ml of blood. After centrifugation of whole blood at 350 *g* for 10 min, platelet-enriched plasma was collected and further centrifuged at 1000 *g* for 10 min. Pellet was washed twice with a buffer containing 10 mM-Tris and 1 mM-EDTA, pH 7.5. Platelet suspensions in 10 mM-Tris and 1 mM-EDTA were frozen in liquid N_2 and stored at -70°C . Before the assay, the frozen platelet suspension was thawed and homogenized, and this freeze-thawing was repeated twice. This preparation was used for adenylate cyclase and ANF-receptor binding studies.

ANF-receptor binding determination. ^{125}I -ANF binding was determined at 25°C by incubating 20 μl of membranes or cell suspension ($\sim 100 \mu\text{g}$) for 60 min with 10 pM- ^{125}I -ANF-(99–126)-peptide (sp. radioactivity 1000 Ci/mmol) in 200 μl of a reaction mixture consisting of 50 mM-Tris/HCl, pH 7.5, 5 mM- MgCl_2 , 1 mM-aminocaproic acid, bacitracin (1 mg/ml), BSA (4 mg/ml) 0.5 mM-phenylmethanesulphonyl fluoride and various amounts of the competing ligands when used. Non-specific binding was determined by using 0.1 μM unlabelled ANF-(99–126)-peptide. Binding reactions were initiated by the addition of membrane protein. The receptor- ^{125}I -ANF complex was separated from free ^{125}I -ANF by filtration through GF/C filters pretreated with polyethyleneimine. The filters were washed three times with 4 ml of ice-cold Tris/HCl buffer, pH 7.5, and radioactivity was counted in a γ -radiation counter.

Affinity cross-linking of ANF. The affinity cross-linking was performed essentially as described by Leitman *et al.* [12], with slight modification. The platelet suspension was incubated at 25°C for 1 h with 1 nM- ^{125}I -ANF-(99–126)-peptide in the absence or presence of 1 μM unlabelled ANF-(99–126)-peptide for non-specific binding. Disuccinimidyl suberate was added to a final concentration of 0.1 mM, and the cell suspension was further incubated for an additional 30 min at 25°C . The contents were centrifuged at 5000 *g* for 15 min. The pellets were suspended in 400 μl of SDS sample buffer [62.5 mM-Tris/HCl (pH 6.8)/10% (v/v) glycerol/5% (v/v) β -mercaptoethanol/2.3% (w/v) SDS] and boiled for 5 min. The solubilized proteins were electrophoresed using 7.5% separating gels as described previously [20]. The gels were fixed, stained, destained, dried and autoradiographed by exposing to Kodak XAR-5 film as described previously [20].

Pertussis-toxin (PT) treatment. The PT treatment was performed as described previously [20]. The platelet membranes were incubated in a buffer containing 25 mM-glycylglycine, pH 7.5, 1 mM- NAD^+ , 0.4 mM-ATP, 0.4 mM-GTP, 15 mM-thymidine, 10 mM-dithiothreitol, and ovalbumin (0.1 mg/ml) without (control) or with PT (5 $\mu\text{g}/\text{ml}$) (treated) for 30 min at 30°C . The platelet membranes were washed two or three times with 10 mM-Tris/1 mM-EDTA buffer, pH 7.5, and finally suspended in the same buffer for adenylate cyclase activity determination. As has been demonstrated previously in aorta [20], preincubation of the platelet membranes at 30°C for 30 min in the absence or presence of PT also resulted in a significant loss of enzyme activity (~ 40 – 50%) that was independent of the presence of PT in the incubation medium. However, the percentage inhibition of adenylate cyclase by ANF remained un-

changed (results not shown). The observed decrease in the enzyme activity during preincubation may be attributed to the instability of the enzyme, because the enzyme adenylate cyclase has been shown to lose its activity exponentially with the time of preincubation at 30°C or 37°C [21].

Adenylate cyclase activity determination. Adenylate cyclase activity was determined by measuring cyclic $[\alpha\text{-}^{32}\text{P}]\text{AMP}$ formation from $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ as described previously [8,20]. The typical assay medium contained 50 mM-glycylglycine, pH 7.5, 0.5 mM- MgATP , $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ $[(1\text{--}1.5) \times 10^6 \text{ c.p.m.}; \text{sp. radioactivity } 30 \text{ Ci/mmol}]$, 5 mM- MgCl_2 (in excess of the ATP concentration), 0.5 mM-cyclic AMP, 1 mM-3-isobutyl-1-methylxanthine, 0.1 mM-EGTA, 10 μM -GTP[S] and an ATP-regenerating system consisting of 2 mM-creatine phosphate, creatine kinase (0.1 mg/ml) and myokinase (0.1 mg/ml) in a final volume of 200 μl . Incubations were initiated by the addition of the particulate fraction (30–70 μg) to the reaction mixture, which had been thermally equilibrated for 2 min at 37°C . Reactions were conducted in triplicate for 10 min at 37°C . Reactions were terminated by the addition of 0.6 ml of 120 mM-zinc acetate. Cyclic AMP was purified by co-precipitation of other nucleotides with ZnCO_3 by the addition of 0.5 ml of 144 mM- Na_2CO_3 and subsequent chromatography by the double-column system described by Salomon *et al.* [22]. To investigate the effect of amiloride treatment on the ANF-mediated inhibition of adenylate cyclase, the platelet membranes were preincubated at 37°C for 10 min without or with 100 μM -amiloride and various concentrations of ANF. This concentration of amiloride has been shown (1) to attenuate completely the inhibitory effects of hormones on adenylate cyclase activity and (2) to inhibit the labelling of PT-catalysed ADP-ribosylation of the inhibitory guanine-nucleotide-regulatory protein G_i [23]. After 10 min preincubation, the adenylate cyclase reaction was initiated with the addition of reaction mixture to the membranes. The reactions were conducted in triplicate for 10 min at 37°C and were terminated as described above. Protein was determined essentially as described by Lowry *et al.* [24], with crystalline BSA as standard.

Determination of cyclic AMP levels. Cyclic AMP levels in platelets were determined by radioimmunoassay as described previously [17] by using a radioimmunoassay kit from du Pont (Mississauga, Ontario, Canada).

RESULTS

Binding of ^{125}I -ANF to rat platelet membranes

The presence of ANF receptors in human platelets has been demonstrated previously [18]. To investigate whether ANF-binding sites are also present in rat platelets, the binding activity was studied using ^{125}I -ANF-(99–126)-peptide. As illustrated in Fig. 1, ^{125}I -ANF binds to rat platelet membranes in a saturable manner. Computer analysis of the saturation binding of labelled ANF over the concentration range studied revealed a single class of saturable high-affinity binding sites with a dissociation constant (K_d) of $125 \pm 16 \text{ pM}$ and a receptor density (B_{max}) of $120 \pm 13 \text{ pm}$ ($n = 3$).

The specificity of binding of ^{125}I -ANF to rat platelet membranes was studied by competitive displacement of radiolabel by unlabelled peptides. As shown in Fig. 2, the binding of ^{125}I -ANF was inhibited in a concentration-dependent manner by ANF-(99–126)-peptide, ANF-(101–126)-peptide, ANF-(103–123)-peptide and ANF-(103–126)-peptide. However, ANF-(99–126)-peptide, ANF-(103–123)-peptide and ANF-(101–126)-peptide were almost equipotent ($\text{IC}_{50} = 0.5$ – 1 nM) and displaced the binding by about 100, 90 and 75% respectively, whereas ANF-(103–126)-

Fig. 1. Binding of ^{125}I -ANF-(99-126) to rat platelet membranes

(a) Platelet membranes were incubated with increasing amounts of labelled ANF-(99-126)-peptide at 25 °C for 60 min in the absence (●) or in the presence (■) of 1 μM unlabelled ANF-(99-126)-peptide as described in the Experimental section. Specific binding (▲) is the difference between two curves. Specific binding in the Scatchard plot (b) was determined with the LIGAND program after fitting the binding data. B_{max} is the maximum number of binding sites; K_d is the dissociation constant. Each point represents the mean of triplicate determinations from a single experiment. Data from three separate independent experiments are given in the Results section.

Fig. 2. Competition for ^{125}I -ANF binding to rat platelet membranes by unlabelled ANF and ANF-derived peptides

Platelet membranes were incubated at 25 °C for 60 min with 10 pM ^{125}I -ANF and increasing concentrations of unlabelled peptides as described in the Experimental section. The binding curves are derived from the specific binding data from at least three separate experiments: ●, ANF-(99-126)-peptide; ▲, ANF-(101-126)-peptide; ○, ANF-(103-123)-peptide; ■, ANF-(103-126). The specific binding normalized to 100% was 4.2 fmol/mg of protein. Total binding was 1504 c.p.m., and binding in the presence of 10^{-6} M-ANF-(99-126)-peptide was 581 c.p.m.

peptide was the least potent ($\text{IC}_{50} \sim 10 \text{ nM}$) and displaced the binding by about 50%.

Effect of ANF on adenylate cyclase activity and cyclic AMP levels

In order to investigate whether the ANF receptors present in rat platelets are functional and are coupled to adenylate cyclase system, the effects on adenylate cyclase activity of various analogues of ANF which were used for binding studies were examined. As shown in Fig. 3, all the analogues of ANF inhibited adenylate cyclase activity in platelets in a concentration-dependent manner with the order of potency of ANF-(103-123)-peptide > ANF-(99-126)-peptide > ANF-(101-126)-

Fig. 3. Effect of ANF and ANF-derived peptides on adenylate cyclase activity in rat platelet membranes

Adenylate cyclase activity was determined in the absence or presence of various concentrations of ANF-(103-123)-peptide (○), ANF-(99-126)-peptide (●), ANF-(101-126)-peptide (▲), or ANF-(103-126)-peptide (■) as described in the Experimental section. Values are means $\pm$ S.E.M. for three separate experiments.

peptide > ANF-(103-126)-peptide. ANF-(103-123)-peptide and ANF-(99-126)-peptide inhibited the enzyme activity by about 50–55%, with an apparent K_i between 0.1 and 0.5 nM, whereas ANF-(101-126)-peptide inhibited the enzyme activity by about 35%, with an apparent K_i between 1 and 3 nM. On the other hand ANF-(103-126)-peptide was the least potent ($K_i \sim 10 \text{ nM}$) and inhibited the adenylate cyclase activity by about 30%. In addition, C-ANF₄₋₂₃, a ring-deleted analogue of ANF [des-Gln¹⁸-Ser¹⁹-Gln²⁰-Leu²¹-Gly²²-ANF-(4-23)-peptide-NH₂], which binds with high affinity to ANF-R2 receptor only [16] and inhibits the adenylate cyclase/cyclic AMP system [17], was also able to inhibit adenylate cyclase activity in rat platelets. The maximal inhibition observed was about 55%, with an apparent K_i of about 10^{-8} M (results not shown). In addition, the effect of ANF-(99-126)-peptide on cyclic AMP levels was also studied, and the results are shown in Table 1. As reported previously for other tissues [8,17], ANF also decreased cyclic AMP levels in rat platelets. The maximal inhibition observed was between 40 and 50%.

Table 1. Effect of ANF-(99-126)-peptide on cyclic AMP levels in rat platelets

Cyclic AMP levels in platelets were determined as described in the Experimental section. Platelets were preincubated with 1 mM-isobutylmethylxanthine for 5 min at 37 °C, ANF-(99-126)-peptide was added to the platelets at different concentrations and were further incubated for 10 min. The reaction was stopped by the addition of 12% trichloroacetic acid (final concn. 6%), and cyclic AMP levels in the supernatant were determined by using a radioimmunoassay kit from du Pont. Values are means $\pm$ S.E.M. for three separate experiments.

[ANF] (M)	Cyclic AMP level (pmol/mg of protein)
None	146 $\pm$ 6.5
10 ⁻¹⁰	115.2 $\pm$ 4
10 ⁻⁸	104 $\pm$ 0.5
10 ⁻⁶	82 $\pm$ 14

Fig. 4. Dependence on guanine nucleotide of ANF-mediated inhibition of adenylate cyclase in rat platelet membranes

Adenylate cyclase activity was determined in the presence of various concentrations of GTP[S] alone (●) or in combination with 0.1 μ M-ANF-(99-126)-peptide (▲). Values are means $\pm$ S.E.M. for three separate experiments. The inset shows the percentage inhibition of adenylate cyclase by ANF at various concentrations of GTP[S].

The inhibitory effect was also dependent on the presence of guanine nucleotides, as shown in Fig. 4. GTP[S] showed a biphasic effect on adenylate cyclase: stimulation at lower concentrations and inhibition at higher concentrations. This differential effect of GTP[S] may be attributed to its interaction with stimulatory (G_s) and inhibitory (G_i) guanine-nucleotide-regulatory proteins respectively, as has been reported in several other tissues [20,25]. ANF-(99-126)-peptide in the absence of exogenously added GTP[S] inhibited the enzyme activity to a small extent ($\sim 20\%$), as shown clearly in the inset to Fig. 4; however, this inhibition was increased in the presence of increasing concentrations of GTP[S]. The maximal inhibition ($\sim 70\%$) was observed in the presence of 5–10 μ M-GTP[S], and beyond that concentration the ANF-induced inhibition was suppressed. The similar suppressive effect of higher concentration of GTP[S] on ANF-mediated inhibition has been reported in the anterior pituitary gland [26].

Fig. 5. (a) Effect of amiloride on ANF-induced inhibition of adenylate cyclase in rat platelet membranes and (b) effect of PT on ANF-mediated inhibition of adenylate cyclase in rat platelet membranes

(a) Platelet membranes were preincubated without (control) or with 100 μ M-amiloride (treated) at 37 °C for 10 min and were used for adenylate cyclase activity determination. Adenylate cyclase activity was determined in the absence or presence of various ANF-(99-126)-peptide concentrations in control (●) and amiloride-treated (▲) platelet membranes in the presence of 10 μ M-GTP[S]. Values are means $\pm$ S.E.M. for three separate experiments. Basal enzyme activities in control and amiloride-treated membranes were 300 $\pm$ 23 and 390 $\pm$ 17 pmol of cAMP/10 min per mg of protein respectively. (b) Platelet membranes were treated without (control) or with PT (treated) as described in the Experimental section. Adenylate cyclase activity was determined in the absence or presence of various concentrations of ANF-(99-126)-peptide in control (●) and PT-treated (▲) platelet membranes as described in the Experimental section in the presence of 10 μ M-GTP[S]. Values are means $\pm$ S.E.M. for three separate experiments. The basal enzyme activities in control and PT-treated platelet membranes were 400 $\pm$ 25 and 521 $\pm$ 33 pmol of cyclic AMP/10 min per mg of protein respectively.

Effect of amiloride and PT on ANF-mediated inhibition of adenylate cyclase

We have recently shown that amiloride interacts with G_i -protein and results in the attenuation of the inhibitory responses of hormones on adenylate cyclase activity in rat aorta and anterior pituitary [23]. In the present studies we have used amiloride in addition to PT to investigate the involvement of G_i -protein in the coupling of ANF receptors to adenylate cyclase in rat platelets. Fig. 5 shows that ANF-mediated inhibition of adenylate cyclase was completely abolished by amiloride (Fig.

Fig. 6. Effect of ANF on basal and stimulatory responses of various agonists on adenylate cyclase activity in rat platelet membranes

Adenylate cyclase activity was determined in the absence (Basal) or presence of 10 μ M-NECA, 1 μ M-PGE₁, 50 μ M-isoprenaline (ISO) and 50 μ M-forskolin (FSK), either alone ($\square$) or in combination with 0.1 μ M-ANF-(99–126)-peptide ($\blacksquare$). Values are means $\pm$ S.E.M. for three separate experiments.

Fig. 7. Autoradiogram of SDS/PAGE of rat platelets after binding with ¹²⁵I-ANF-(99–126)-peptide and cross-linking with disuccinimidyl suberate

Platelets were incubated with 1 nM-¹²⁵I-ANF-(99–126)-peptide for 1 h at room temperature in the absence (lane 1) or presence (lane 2) of 1 μ M unlabelled ANF-(99–126)-peptide. The platelets were then cross-linked by adding disuccinimidyl suberate to a final concentration of 0.1 mM for 30 min. The platelets were solubilized with SDS sample buffer and subjected to SDS/PAGE as described in the Experimental section.

5a) and PT (Fig. 5b) treatments, indicating that, in platelets also, the ANF receptors are coupled to adenylate cyclase through a G_i-protein and supports the results shown in other tissues [20,27]. In addition, amiloride, as was shown previously for zona-glomerulosa cells [28], also enhanced the ANF binding in platelets by about 2-fold (results not shown), indicating that this may be the characteristic feature of amiloride action on ANF binding.

Various hormones have been reported to play an important role in platelet functions through the stimulation of the adenylate cyclase/cyclic AMP system [29]. It was of interest to examine whether ANF can modulate the stimulatory responses of hormones on adenylate cyclase activity. Fig. 6 demonstrates that NECA, prostaglandin E₁, isoprenaline and forskolin stimulated adenylate cyclase activity to various extents, and ANF (10⁻⁷ M) inhibited the stimulatory effect of all these agonists; however, the stimulations were not completely abolished.

Cross-linking studies

To determine whether rat platelets possess multiple binding sites for ANF, ¹²⁵I-ANF-(99–126)-peptide was covalently cross-linked to intact platelets with the bifunctional reagent disuccinimidyl suberate in the absence or presence of 1 μ M unlabelled ANF-(99–126)-peptide, and the cellular proteins were then separated by SDS/PAGE. Fig. 7 demonstrates that ¹²⁵I-ANF labelled predominantly one binding site of M_r about 66000, which was completely displaced by unlabelled ANF. About 60–70 % inhibition in the labelling of this protein band was also observed by 10 nM unlabelled ANF (results not shown). These results indicate that rat platelets possess only the type of ANF receptors designated 'ANF-R2' [12].

DISCUSSION

In the present study we demonstrate the presence of ANF-binding sites in rat platelet membranes. Unlike adrenal zona-glomerulosa cells, where both high- and low-affinity binding sites for ANF have been shown to be present [30], the binding of ¹²⁵I-ANF to rat platelet membranes revealed a single class of specific binding sites. The presence of single class of ANF-binding sites has also been reported in several tissues, including human platelets [18,31]. The binding affinity ($K_d \sim 120$ pM) observed in rat platelet membranes was found to be over a similar range to that demonstrated in several other tissues [18,31,32]. In addition, the unlabelled peptides ANF-(99–126)-peptide, ANF-(101–126)-peptide, ANF-(103–123)-peptide and ANF-(103–126)-peptide inhibited the binding of the labelled ANF-(99–126)-peptide in rat platelets in a manner similar to that reported in other tissues, including human platelets [18].

ANF at physiological concentrations has been reported to inhibit adenylate cyclase and cyclic AMP levels in numerous tissues [4,7–11]. The inhibition of this signal-transduction system by ANF may be one of the important mechanisms through which ANF exerts its physiological responses, such as inhibition of aldosterone secretion [33], inhibition of renin release [33], inhibition of vasopressin release [34] and inhibition of progesterone secretion [36]. In the present study we demonstrate that various analogues of ANF that inhibited the binding of radio-labelled ANF also inhibited the adenylate cyclase activity in an analogous manner: ANF-(103–123)-peptide was the most potent, and ANF-(103–126)-peptide the least potent. The maximal inhibitions observed were between 30 and 55 %, which were similar to those reported for other tissues, such as glomeruli, vascular smooth-muscle cells and pituitary [4,7,9]. The apparent K_i of inhibition of adenylate cyclase by the ANF analogues and the IC₅₀ required to inhibit ¹²⁵I-ANF binding were in the same range, i.e. between 0.1 and 1 nM for ANF-(99–126)-peptide, ANF-(101–126)-peptide, ANF-(103–123)-peptide and about 10 nM for ANF-(103–126)-peptide, and indicate that the receptor occupancy results in the activation of the adenylate cyclase/cyclic AMP pathway.

As reported in other tissues [7,8,27], the inhibition of adenylate cyclase by ANF in rat platelets was also dependent on the presence of guanine nucleotides, indicating that ANF receptors are coupled to adenylate cyclase system by guanine-nucleotide-regulatory protein. The involvement of G_i in the coupling of ANF receptors to adenylate cyclase was demonstrated previously [20,27]. In the present studies, amiloride and PT were also able to attenuate the ANF-mediated inhibition of adenylate cyclase, which strongly suggests that ANF receptors in platelets are also coupled to adenylate cyclase system through a G_i-protein.

Cyclic AMP plays an important role in a variety of physiological functions, including platelet aggregation. Various hormones which stimulate the adenylate cyclase/cyclic AMP system

are inhibitors of platelet aggregation [29]. In the present studies, we demonstrate that NECA, isoprenaline, prostaglandin E_1 and forskolin stimulated adenylate cyclase to various extents, and ANF, as in other tissues [4,7–11], inhibited the stimulatory effects of all these agents in platelets also. However, contradictory effects of ANF on platelet aggregation have also been demonstrated. ANF has been shown to have no effect on platelet aggregation, whereas Loeb & Gaer [37] have recently reported that ANF potentiated the platelet aggregation induced by thrombin, ADP and adrenaline (epinephrine). Taken together, these results may indicate that the inhibition of adenylate cyclase activity by ANF may be one of the possible mechanisms responsible for the potentiation of platelet aggregation.

ANF has also been demonstrated to stimulate particulate guanylate cyclase activity and cyclic GMP levels in a variety of tissues [2], with the exception of platelets, which have been reported to be devoid of particulate guanylate cyclase activity [19]. It is also known that some, but not all, the physiological effects of ANF are mediated through stimulation of the guanylate cyclase/cyclic GMP system. By using affinity cross-linking studies, or photoaffinity-labelling techniques, two classes of ANF receptors have been shown to be present in various tissues [12,13]. These receptors have M_r values of 130000–140000 and 66000 and are designated 'ANF-R1' and 'ANF-R2/C' [12,16] respectively. The 130000- M_r receptors, ANF-R1, are known to be coupled to guanylate cyclase activity, whereas ANF-R2/C receptors are coupled to the adenylate cyclase/cyclic AMP system [17]. As expected, in the present studies, by using the affinity cross-linking labelling technique, we demonstrate specific predominant labelling of a single protein band of M_r about 66000 in rat platelets, which was completely inhibited in the presence of unlabelled ANF, indicating that rat platelets lack ANF-R1 receptors and possess only ANF-R2 receptors. These findings accord very well with a previous report indicating a lack of ANF effect on cyclic GMP levels and the absence of particulate guanylate cyclase in platelets [19]. Since ANF inhibits adenylate cyclase activity in the platelet membranes, it clearly indicates that the ANF-R2 receptors are associated with the adenylate cyclase/cyclic AMP signal-transduction system and further supports our previous results [17]. Recently Tseng *et al.* [38] have demonstrated that ANF inhibited cyclic AMP generation and thyroglobulin secretion without increasing cyclic GMP levels in human cultured thyroid cells, which have only ANF-R2 receptor subtypes. In addition C-ANF_{4–23}, which interacts with ANF-R2 receptors, has also been reported to inhibit cyclic AMP levels and adrenergic neurotransmission independent of guanylate cyclase in nerve-growth-factor-treated pheochromocytoma cells [39]. We have also reported recently that C-ANF_{4–23} inhibited luteinizing-hormone-stimulated progesterone secretion in mouse Leydig tumour cells [17]. Taken together, it may be suggested that the potentiating effect of ANF on adrenaline, thrombin and ADP on platelet aggregation may be mediated through ANF-R2 receptors present in platelets and which are associated with the adenylate cyclase/cyclic AMP signal-transduction system.

ANF has also been shown to inhibit the formation of inositol phosphates [40], an effect that can be mimicked by 8-bromo cyclic GMP and may thus be mediated through the occupancy of the ANF-R1 receptors. On the other hand, recently, ANF has also been reported to stimulate the PtdIns turnover in the presence of guanine nucleotides in bovine aortic smooth-muscle cells, which has been demonstrated to be through its interaction with ANF-R2 receptors [40]. Thus it is possible that ANF-R2 receptors may be coupled to two different intracellular messengers, cyclic AMP and PtdIns turnover, or there may exist a 'cross-talk' between these two second messengers. It is well known that the hormones such as angiotensin II, vasopressin

and acetylcholine, which inhibit the adenylate cyclase/cyclic AMP system, also stimulate PtdIns turnover [41], and the hormones which increase cyclic AMP are inhibitors of PtdIns turnover [42]. In the light of these observations, it is possible that the decrease in cyclic AMP by ANF may be responsible for stimulating PtdIns turnover. In other words, the stimulation of PtdIns turnover by ANF in cultured vascular cells [40] may be the secondary event and mediated through the adenylate cyclase/cyclic AMP system to which are coupled the ANF-R2 receptors.

In conclusion, our present studies demonstrate that rat platelets only have ANF-R2 subtype receptors, which are coupled to the adenylate cyclase/cyclic AMP signal-transduction system. Whether activation of this signal-transduction system by ANF is associated with PtdIns turnover requires thorough investigation.

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