Identification of Multiple Binding Sites for Atrial Natriuretic Factor by Affinity Cross-linking in Cultured Endothelial Cells*

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In a previous study, we found that atriopeptin I was much weaker (EC50 > 500 nM) than atrial natriuretic factor (ANF-(8-33)) (EC50 = 0.3 nM) at increasing cyclic GMP on cultured endothelial cells. In this study, we used the cross-linking reagent disuccinimidyl suberate to investigate whether the differences in activity were due to the presence of multiple ANF receptors. When 98% of the ANF-binding sites on endothelial cells were occupied by tyrosine-atriopeptin I after cross-linking, there was no difference in the concentration-response curve to ANF-(8-33) with regard to cyclic GMP accumulation. In contrast, when 96% of the binding sites were occupied by cross-linked ANF-(8-33), a 60% decrease in the maximal cyclic GMP response was observed after the readdition of ANF-(8-33). These results suggest that ANF-(8-33) is binding to an additional site that atriopeptin I does not effectively bind. Affinity cross-linking of 125I-ANF to intact endothelial cells resulted in the labeling of two sites of $M_r$ ~66,000 and ~130,000. Approximately 94% of the 125I-ANF binding sites had $M_r$ ~66,000. Labeling of this site was inhibited by both tyrosine-atriopeptin I ($K_I = 0.9$ nM) and ANF-(8-33) ($K_I = 0.09$ nM). Although 0.1 μM tyrosine-atriopeptin (AP I) inhibited labeling of the 66,000-dalton site to nearly the same degree as ANF-(8-33), it produced only a 4-fold increase in cyclic GMP compared to a 400-fold increase with ANF-(8-33). These results suggest that the 66,000-dalton site is not coupled to guanylate cyclase and cyclic GMP formation. Tyrosine-AP I ($K_I > 10$ nM) was much weaker at competing for the 130,000-dalton site than ANF-(8-33) ($K_I = 0.075$ nM). Because the EC50 for cyclic GMP stimulation for tyrosine-AP I (>100 nM) and ANF-(8-33) (0.4 nM) is closer to the $K_I$ values for the 130,000-dalton protein, this site probably mediates the marked stimulation of cyclic GMP. Our results demonstrate that endothelial cells contain two binding sites for ANF-(8-33) and suggest that only the less abundant site ($M_r$ ~130,000) is the receptor coupled to the activation of guanylate cyclase.

Atrial natriuretic factor (ANF3)) is a recently discovered group of small peptide hormones that are synthesized and secreted from the atria (1-3). The administration of ANF produces diuresis, natriuresis, and hypotension in vivo (1-5). The precise mechanisms whereby ANF elicits these physiological effects is unknown. ANF has been reported to selectively activate the particulate form of guanylate cyclase (6-8), stimulate cyclic GMP formation (6, 7, 9, 10), increase cyclic GMP-dependent protein kinase activity (11), and inhibit adenylyl cyclase (12) in a variety of tissues. ANF receptors have been identified in membranes from rabbit aorta and kidney (13), bovine adrenal glomerulosa (14) and cultured vascular endothelial (15, 16), and smooth muscle (10, 15, 17) cells. In previous studies, we compared the binding of several atrial natriuretic peptides with their ability to increase cyclic GMP formation in bovine aortic endothelial (16) and smooth muscle (17) cells. Our results showed that the 21-amino acid peptide atriopeptin I was very effective at competing for 125I-ANF-binding sites, but was a very weak stimulator of cyclic GMP production (16, 17). In endothelial cells, 1 pm ANF-(8-33) increases cyclic GMP by 5-fold, and at 10 nM an approximate 800-fold stimulation of cyclic GMP occurs in the presence of an inhibitor of phosphodiesterases. In contrast, atriopeptin I is a much less effective agonist. It begins to increase cyclic GMP at 10 nM and produces only a 7-fold increase in cyclic GMP at 0.1 μM. Because atriopeptin I was unable to antagonize the stimulation of cyclic GMP accumulation induced by the more potent atrial peptide ANF-(8-33), we proposed that endothelial cells may contain multiple ANF receptors (16). Typically, the identification of multiple receptors is established with selective agonists and antagonists for the specific receptor subtypes. However, no such selective probes are available for ANF receptors. In order to investigate further the possible existence of multiple ANF receptors in endothelial cells, we used affinity cross-linking techniques (18) in combination with cyclic GMP measurements. Our results with this novel approach provide definitive evidence for the existence of two ANF-binding sites of $M_r$ ~66,000 and ~130,000. Furthermore, our data indicate that only the less abundant site ($M_r$ ~130,000) is coupled to guanylate cyclase and cyclic GMP formation.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin/streptomycin, and Hanks' balanced salt solution

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The abbreviations used are: ANF-(8-33), atrial natriuretic factor (Arg16-Tyr33); AP I, atriopeptin I; tyrosine-AP I, tyrosine-atriopeptin I (Tyr,Ser27, Ser32); DSS, disuccinimidyl suberate; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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(HBSS) were purchased from Grand Island Biological Co. Calf serum was purchased from Sterile Systems Inc. Tissue culture dishes were from Falcon. Disuccinimidyl suberate was purchased from Pierce Chemical Co. Atriopeptin I, tyrosine-atriopeptin I, and atrial natriuretic factor (8-33) were synthesized at Peninsula Laboratories, Inc. All other reagents were obtained as previously described (19, 20).

Cell Culture—Endothelial cells were prepared and cloned from bovine aorta as described by Longenecker et al. (21). The cells were maintained and subcultured in DMEM containing 10% calf serum, 584 μg/ml glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells were incubated at 37 °C in a 5% CO₂ humidified incubator.

Iodination of Atrial Peptides—In order to iodinate atriopeptin I, a tyrosine residue was added to the amino-terminal end of the native atriopeptin I. The tyrosine-atriopeptin I analog competed for 125I-tyrosine-AP I and 125I-ANF-(8-33) binding sites and exhibited similar effects on cyclic GMP accumulation as the native atriopeptin I. Tyrosine-AP I and ANF-(8-33) (0.3 nmol) were incubated with 500–1000 μCi of Na[125]I for 15 min at room temperature in the presence of IOODO-GEN (22). Unbound iodide was separated from the labeled peptide with a Sep-Pak C₁₈ column (23). The specific radioactivity of the atrial peptides was 700–1400 Ci/mmol.

Affinity Cross-linking Studies—Confluent endothelial cells in 35-mm dishes were washed three times with 2 ml of HBSS containing 10 mM HEPES, pH 7.3. The cells were incubated in 1 ml of HBSS containing either 0.1 μM tyrosine-AP I or ANF-(8-33) for 1 h at room temperature. One milliliter of HBSS containing 0.2 mM DSS (dissolved in dimethyl sulfoxide) was added, and the cells were incubated an additional 30 min at room temperature. The cells were washed four times with HBSS containing 2 mg/ml bovine serum albumin. The cross-linked cells were then used for either radioavidin binding or cyclic GMP response studies.

RESULTS

Intact endothelial cell monolayers were exposed to 0.1 μM tyrosine-atriopeptin I for 1 h. The bound tyrosine-atriopeptin I was cross-linked to ANF-binding sites by the addition of 0.1 mM DSS. To determine the efficiency of cross-linking, the cell cultures were then examined for their ability to bind 125I-tyrosine-atriopeptin I. Table I (Experiment 1) shows that cultures exposed to 0.1 μM tyrosine-atriopeptin I (Tyr-AP I – DSS), but not cross-linked with DSS, bound approximately 85% of 125I-tyrosine-atriopeptin I as compared to the specific binding in the control cells. In contrast, there was a marked decrease in 125I-tyrosine-AP I binding to the cells cross-linked with DSS (Tyr-AP I + DSS). After subtracting nonspecific binding, it was established that approximately 98% of the ANF-binding sites were occupied with cross-linked tyrosine-AP I. Fig. 1A shows the effect of increasing concentrations of ANF-(8-33) on cyclic GMP accumulation in cells cross-linked with tyrosine-AP I. The basal cyclic GMP in the cross-linked cells was 0.4 pmol/10⁶ cells, which was 2.5-fold higher than the control value. Although 98% of the binding sites were occupied by tyrosine-AP I, there was no decrease in the maximal cyclic GMP response, nor was there a shift in the ANF-(8-33) concentration-response curve.

Endothelial cells were also cross-linked with ANF-(8-33), which is a very potent stimulator of cyclic GMP accumulation. Table I (Experiment 2) demonstrates that cross-linking with ANF-(8-33) resulted in a large decrease in the subsequent binding of 125I-ANF-(8-33). In this case, approximately 96% of the binding sites were bound by cross-linked ANF-(8-33). Fig. 1B shows that endothelial cells cross-linked with ANF-(8-33) had a basal level of cyclic GMP of 4.2 pmol/10⁶ cells, which was 10-fold higher than cells cross-linked with tyrosine-AP I. The subsequent addition of increasing concentrations of ANF-(8-33) resulted in a small increase in cyclic GMP levels in the cross-linked cells to a maximal value of 9 pmol/10⁶ cells with 10 nM ANF-(8-33). The cyclic GMP levels in the control cells began to exceed the value in the cross-linked cells at 1 nM ANF-(8-33), and with 0.1 μM ANF-(8-33) there was a 2.2-fold increase in cyclic GMP levels. These results demonstrate that, unlike tyrosine-AP I, cross-linking with ANF-(8-33) is capable of antagonizing the subsequent stimulation of cyclic GMP accumulation by ANF-(8-33). The possibility that the diminished response to ANF-(8-33) may be due to the desensitization of guanylate cyclase by ANF-(8-33) was considered. In order to examine this possibility, endothelial cells were pretreated with 0.1 μM ANF-(8-33) for...
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Fig. 1. A, effect of cross-linking with tyrosine-atriopeptin I on ANF-(8-33)-induced stimulation of cyclic GMP in bovine aortic endothelial cells. Cells were incubated with 0.1 μM tyrosine-AP I for 1 h, and cultures were then treated with dimethyl sulfoxide (control, ○) or 0.1 mM DSS (cross-linked, □) as described under Experimental Procedures. After washing, all cell cultures were incubated with increasing concentrations of ANF-(8-33) for 3 min at 37 °C in the presence of 0.5 mM isobutylmethylxanthine. The intracellular cyclic GMP levels were measured by radioimmunoassay. The basal cyclic GMP value in the control and cross-linked cells was 0.16 and 0.44 pmol/10^6 cells, respectively. Each determination represents the mean of triplicate dishes from a representative experiment.

B, effect of cross-linking with ANF-(8-33) on ANF-(8-33)-induced stimulation of cyclic GMP in bovine aortic endothelial cells. Endothelial cell cultures were exposed to 0.1 μM ANF-(8-33) for 1 h. The cells were then treated with dimethyl sulfoxide (control, ○) or 0.1 mM DSS (cross-linked, □) as described under Experimental Procedures. After washing, all cultures were then incubated with increasing concentrations of ANF-(8-33) for 3 min at 37 °C in the presence of 0.5 mM isobutylmethylxanthine. The basal cyclic GMP value in the control and cross-linked cells was 0.075 and 4.2 pmol/10^6 cells, respectively. Each determination represents the mean of triplicate dishes from a representative experiment.

1 h, washed extensively, and then re-exposed to increasing concentrations of ANF-(8-33). We found that pretreatment of endothelial cells with ANF-(8-33) had no effect on the concentration-response curve with readdition of ANF-(8-33), suggesting that ANF-(8-33) does not promote the desensitization of guanylate cyclase under these conditions (data not shown).

To determine if endothelial cells possess multiple binding sites for ANF-(8-33) as suggested by the physiological studies, intact monolayers were cross-linked with 125I-ANF-(8-33) in the presence of increasing concentrations of either tyrosine-AP I or ANF-(8-33) and the cellular proteins were then separated by SDS-polyacrylamide gel electrophoresis. The autoradiographs show that 125I-ANF-(8-33) labeled two binding sites (Fig. 2, A and B). The predominant binding site had a molecular size of approximately 66,000 daltons, whereas the second site labeled had a molecular size of approximately 130,000 daltons. By scanning autoradiographs with a densitometer, it was determined that the 130,000-dalton site represented 6.14 ± 0.44% (S.D., n = 5) of 125I-ANF-(8-33)-binding sites. Furthermore, when the bands were excised from the gel, it was determined that the 130,000-dalton band contained 6.6 ± 0.56% (S.D., n = 5) of the radioactivity that was specifically incorporated into the 66,000- and 130,000-dalton binding sites (data not shown).

Tyrosine-atriopeptin I was compared to ANF-(8-33) for its ability to inhibit 125I-ANF-(8-33) labeling of the 66,000- and 130,000-dalton sites (Fig. 2, A and B). The degree of inhibition was quantitated by scanning the autoradiographs shown in Fig. 2 (A and B) with a densitometer. Fig. 2 (C and D) shows that both tyrosine-AP I and ANF-(8-33) effectively inhibit labeling of the 66,000-dalton site. For this site, the Kᵢ of tyrosine-AP I was approximately 10-fold higher than the Kᵢ of ANF-(8-33) (Table II). Fig. 2C shows that 0.1 μM tyrosine-AP I inhibited labeling of the 66,000-dalton site to nearly the same extent as 0.1 μM ANF-(8-33). However, at this concentration, tyrosine-AP I produced only a 4-fold increase in cyclic GMP compared to more than a 1,000-fold increase with ANF-(8-33) (Fig. 2C and D). These results demonstrate that ANF binding to the 66,000-dalton site is not associated with an increase in cyclic GMP formation.

Fig. 2A shows that tyrosine-AP I was less effective at inhibiting the labeling of the 130,000-dalton site when compared to the 66,000-dalton site and much weaker than ANF-(8-33) at inhibiting labeling of the 130,000-dalton site. At this site, the Kᵢ for tyrosine-AP I was at least 150-fold greater than the Kᵢ for ANF-(8-33) (Table II). In contrast to tyrosine-AP I, the Kᵢ for ANF-(8-33) at the 130,000-dalton site was similar to its Kᵢ for the 66,000-dalton site. Furthermore, the EC₅₀ for cyclic GMP stimulation was similar to the Kᵢ of ANF-(8-33) for these two sites. These results demonstrate that the 66,000-dalton site has a greater affinity for tyrosine-AP I than the 130,000-dalton site and that both of these sites have a similar affinity for ANF-(8-33).

DISCUSSION

In previous studies, we found that atriopeptin I was very effective at competing for 125I-ANF-binding sites when compared to ANF-(8-33), but was a weak stimulator of cyclic GMP production in endothelial (16) and smooth muscle (17) cells. Several possible explanations may account for the apparent disparity between the binding and cyclic GMP responses. Assuming that these cells possess a single receptor for ANF, as suggested by a linear Scatchard plot (16, 17), these results could be due to differences in binding affinity for the two peptides. This possibility, however, is unlikely since the Kᵢ values for AP I and ANF-(8-33) were similar when the binding of 125I-ANF-(8-33) was examined (16, 17).

In endothelial and smooth muscle cells, the Kᵢ for AP I was only 6-fold higher than the Kᵢ for ANF-(8-33) despite an EC₅₀
for cyclic GMP stimulation that was 100-1500-fold higher for AP I than ANF-(8-33) (16, 17). Furthermore, endothelial cells cross-linked with ANF-(8-33) had a 10-fold higher basal cyclic GMP level than those cells cross-linked with tyrosine-AP I. These results suggest that ANF-(8-33) is much more effective than tyrosine-AP I in activating guanylate cyclase when both are bound to ANF-binding sites.

A second possible explanation for these findings is that AP I readily binds to a single class of ANF receptors, but does not activate guanylate cyclase as effectively as ANF-(8-33) (i.e. the coupling to guanylate cyclase activation is different and ineffective). In order to address this possibility, we cross-linked cells with both tyrosine-AP I and ANF-(8-33) and then measured the cyclic GMP response to ANF-(8-33). Our results demonstrated that, when 98% of the binding sites were occupied by tyrosine-AP I, there was no alteration in the concentration-response curve to ANF-(8-33).

These results could occur if there was a large number of spare receptors so that only 2% or less of the total ANF-binding sites are required for a maximal cyclic GMP response. If spare receptors were responsible for the inability of tyrosine-AP I to block the stimulation by ANF-(8-33), we would expect that cross-linking with ANF-(8-33) would produce a similar result. However, when cells were cross-linked with ANF-(8-33), there was a 60% decrease in the maximal cyclic GMP response. This observation suggests that ANF-(8-33) is binding to a second site that tyrosine-AP I does not effectively bind and that this site is coupled to guanylate cyclase activation and increased cyclic GMP formation. It seems apparent that, when the cells are cross-linked with tyrosine-AP I, many of the guanylate cyclase-coupled sites remain vacant, allowing ANF-(8-33) to bind effectively and to activate guanylate cyclase. In contrast, when the cells are cross-linked with ANF-(8-33), both the guanylate cyclase-coupled and uncoupled sites are apparently occupied, leading to an increased basal level of cyclic GMP and a diminished maximal cyclic GMP response after the readdition of ANF-(8-33).
The existence of multiple ANF-binding sites was confirmed by SDS-polyacrylamide gel electrophoresis. Approximately 94% of the sites labeled by 125I-ANF-(8-33) had an Mr, ~66,000. Labeling of this site was decreased by both tyrosine-AP I and ANF-(8-33). The observation that the Mr for tyrosine-AP I was approximately 10-fold higher than the Mr for ANF-(8-33) is consistent with our previous competition binding studies with endothelial cells (16). Our finding that tyrosine-AP I effectively competes with 125I-ANF-(8-33) for the 66,000-dalton binding sites to a similar extent as ANF-(8-33) but does not elicit the large increases in cyclic GMP accumulation suggests that this site is probably not coupled to guanylate cyclase and cyclic GMP formation. If the 66,000-dalton site mediated the increase in cyclic GMP, but AP I was an ineffective agonist at this site, we would have expected it to antagonize the ANF-(8-33)-induced stimulation of cyclic GMP. 125I-ANF-(8-33) also labeled a second site of Mr ~130,000, which comprised approximately 6% of the 125I-ANF-(8-33)-binding sites. With respect to this binding site, ANF-(8-33) was much more effective than tyrosine-AP I at decreasing the labeling of 125I-ANF-(8-33). Whereas the Mr for tyrosine-AP I was 10-fold higher than the Mr for ANF-(8-33) at the 66,000-dalton site, it was over 150-fold higher at the 130,000-dalton site. These findings demonstrate that the 66,000-dalton site has a higher affinity for AP I than the 130,000-dalton site and suggest that AP I may be potentially useful as a relatively selective peptide for this site. These results also suggest that the COOH-terminal amino acids, phenylalanine-arginine-tyrosine, are necessary for effective binding to the 130,000-dalton site, but not to the 66,000-dalton site. Furthermore, we found that 0.1 μM tyrosine-AP I inhibited the binding of 125I-ANF-(8-33) by 35%, but produced only a 4-fold rise in cyclic GMP. In contrast, ANF-(8-33) elicited an approximate 100-fold stimulation of cyclic GMP at a concentration required to inhibit the labeling of the 66,000-dalton site by 35%. This observation and our previous studies (16, 17) showing that atropine II and atropine III increase cyclic GMP to nearly the same extent as ANF-(8-33) suggest that the carboxyl-terminal phenylalanine-arginine are the important residues for coupling the ANF receptor to the activation of guanylate cyclase.

Our studies also showed that the EC50 for cyclic GMP stimulation by tyrosine-AP I is over 250-fold higher than the EC50 for ANF-(8-33). These results demonstrate that the EC50 for cyclic GMP stimulation by tyrosine-AP I and ANF-(8-33) correlates better with the Mr of these peptides for the 130,000-dalton site. We have also cross-linked endothelial cells with 125I-tyrosine-atriopeptin I and found essentially all the radioactivity in the 66,000-dalton protein (data not shown). Taken together, these findings suggest that the 130,000-dalton site is more likely the ANF receptor coupled to guanylate cyclase. Recent work in our laboratory (26) has demonstrated that highly purified particulate guanylate cyclase (Mr, ~120,000) from rat lung exhibits high affinity and specificity binding for ANF-(8-33). These results suggest that the 130,000-dalton binding site in endothelial cells that is coupled to cyclic GMP formation may be particulate guanylate cyclase. However, additional studies are needed to determine the precise relationship between the 130,000-dalton site and guanylate cyclase.

Affinity cross-linking techniques have been also used to identify an ANF-binding site with an Mr, ~130,000 in membranes from rat kidney (27) and bovine adrenal cortex (28, 29). In rabbit aorta membranes, three binding sites for ANF have been identified with molecular sizes of 69,000, 70,000, and 120,000 daltons (30). These differences may reflect multiple ANF receptors or may result from the cellular heterogeneity of the tissue and species examined. From our results, we can conclude that cellular heterogeneity cannot account for the observation of multiple binding sites since cloned endothelial cells were used in these studies. Another advantage of using cloned, intact cells was that we were able to measure a physiological response (cyclic GMP synthesis) to ANF-(8-33) after cross-linking. The use of cross-linking studies coupled with cyclic GMP formation has also allowed us to examine the heterogeneity of ANF-binding sites when specific antagonists are unavailable. By taking this novel approach, we were able to identify multiple ANF-binding sites in endothelial cells and to determine that the binding site with Mr ~130,000 is most likely coupled to guanylate cyclase and cyclic GMP formation. We have also obtained very similar results with cultured bovine smooth muscle and bovine adrenal cortical cells (data not shown). We do not know the relationship of the 66,000-dalton binding site to the 130,000-binding site or its intracellular messenger. However, the approach used in these studies should be useful in identifying its intracellular messenger and possibly the messengers coupled to other hormone receptor systems. In order to facilitate future discussion of these two ANF-binding sites, we suggest that the guanylate cyclase-coupled site (Mr ~130,000) be designated ANF-R1 and the uncoupled site (Mr ~66,000) ANF-R2.

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