AP-811, a novel ANP-C receptor selective agonist

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AP-811 is a derivative of the Phe8-Ile15 region of atrial natriuretic peptide (ANP) and is one of the smallest linear ligands for ANP receptors. The binding and agonist activities of AP-811 have been compared with those of other ANP analogs for the ANP-A and ANP-C receptors. AP-811 binds with a high binding affinity to and is a strong agonist for the ANP-C receptor, indicating that the binding and agonist sites for this receptor are the same or near each other in the ANP sequence. In contrast, AP-811 showed no agonistic effect for the ANP-A receptor, although it could bind to this receptor. Comparing the biological activities of AP-811 with those of other ANP analogs, we propose that the binding and agonist sites for the ANP-A receptor may consist of separate regions of ANP. In conclusion, AP-811 is the smallest C-receptor-selective agonist.

MATERIAL AND METHODS

Materials. AP-811 was synthesized in our laboratory by classical solution techniques and fragment condensation using tert-butyloxycarbonyl (t-Boc) methods (8, 10). Briefly, H-Arg(Tos)-Ile-Asp(Obzl)-Arg(Tos)-NH-(S)-2-methylbutyl was synthesized stepwise from the C-terminus. The resulting compound was coupled with 4-(2-naphthoylamino)phenylacetic acid chloride using 1-hydroxybenzotriazole (HOBT) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSCD) as coupling reagents. After deprotected by anhydrous hydrogen fluoride, the crude AP-811 was purified by Sephadex G-10 gel filtration chromatography.
unlabeled ligands. After incubation, cells were washed twice with NaCl/KCl/Pi containing 0.5 

Buffer A, in the presence of various concentrations of bovine serum albumin (buffer A), and then incubated at 37 °C for 10 min in the same buffer. The reaction was started by adding various ligands followed by incubation at 37 °C for 10 min, and stopped by adding the same volume of ice-cold 12% trichloroacetic acid. After removal of the trichloroacetic acid solution, the cells were washed once with 6% trichloroacetic acid solution. These solutions were combined and centrifuged at 3000 rpm for 30 min at 4 °C, followed by washing the supernatant with two volumes of water-saturated ether three times to remove trichloroacetic acid. Aliquots of the aqueous solution was evaporated and assayed for cAMP or cGMP using RIA kits.

Statistics. All values are expressed as mean ± SEM. Statistical analysis for comparisons within data for the inhibition of cAMP production in rVSMC cells was carried out using the analysis of variance (ANOVA) method with Dunnett’s test (14). Differences were considered to be significant at P < 0.05.

RESULTS

Binding profiles and agonistic effect for the ANP-C receptor

The structures of AP-811, natural natriuretic peptides, and the ANP-C receptor-selective agonists used in the following studies are summarized in Fig. 1. The binding affinities of these compounds for the ANP-C receptor expressed on Hela cells were compared. As shown in Fig. 2, all ligands showed similar high affinities to the ANP-C receptor. The IC50 values of these ligands against 50 pM of [125I]-hANP for binding to this receptor were summarized in the caption of Fig. 2.

It was reported that rVSMC predominantly express the ANP-C receptor as an ANP binding site (15). These cells were used to investigate the agonistic activity of the ANP-C receptor, which was measured as the reduction of cAMP production (16). As shown in Fig. 3, AP-811 significantly inhibits cAMP production in rVSMC, similar to MetSO-hANP, although their potencies are weaker than those in hANP or C-ANF4-23. pBNP partially, but significantly, inhibited adenylate cyclase activity; however, hCNP did not have inhibitory activity of adenylate cyclase, even at 100 nM.

Binding profiles and agonistic effect for the ANP-A receptor

The binding properties of AP-811 and other ANP analogs on the ANP-A receptor in CPAE cells were investigated as shown in Fig. 4. The binding affinities of the ligands for CPAE cells were hANP > pBNP > MetSO-hANP > C-ANF4-23 > AP-811. The IC50 values of these ligands against 50 pM of [125I]-hANP for

Cyclic nucleotide measurement. The measurements of cyclic nucleotide production were carried out as described previously (9). The confluent cell monolayers in 24-well dishes were washed with assay buffer (NaCl/KCl/Pi containing 1 μM thiorphan, 10 μM PMSF, 30 μM leupeptin, 0.5 mM isobutyl methylxanthine and 0.5% bovine serum albumin), and then incubated at 37 °C for 10 min in the same buffer. The reaction was started by adding various ligands followed by incubation at 37 °C for 10 min, and stopped by adding the same volume of ice-cold 12% trichloroacetic acid. After removal of the trichloroacetic acid solution, the cells were washed once with 6% trichloroacetic acid solution. These solutions were combined and centrifuged at 3000 rpm for 30 min at 4 °C, followed by washing the supernatant with two volumes of water-saturated ether three times to remove trichloroacetic acid. Aliquots of the aqueous solution was evaporated and assayed for cAMP or cGMP using RIA kits.

Structures of AP-811, natural natriuretic peptides (hANP, pBNP and hCNP) and ANP-C receptor-selective agonists (MetSO-hANP and C-ANF4-23).

FIGURE 1

Structures of AP-811, natural natriuretic peptides (hANP, pBNP and hCNP) and ANP-C receptor-selective agonists (MetSO-hANP and C-ANF4-23).
binding to this receptor are summarized in the caption to Fig. 4. hCNP did not bind to CPAE cells.

The agonistic effects of AP-811 and other ANP analogs were compared by measuring the ability to stimulate a guanylate cyclase coupled to the ANP-A receptor. Figure 5 shows the concentration dependence of cGMP formation induced by these ligands in CPAE cells expressing the ANP-A receptor. hANP, pBNP and MetSO-hANP stimulated cGMP production in CPAE cells in a dose-dependent fashion up to 250 fold (66 ± 2 fmol/10^5 cells at basal level vs. 16.5 ± 1.1 pmol/10^5 cells when incubated with 10^-5 M of hANP). Their
The biological activities of ANP are mediated by cGMP through the activation of the particulate guanylate cyclase coupled to the ANP receptor (1). Two subclasses of the ANP receptor, the ANP-R\textsubscript{1} and the ANP-R\textsubscript{2} receptors, have been isolated (17, 18). The ANP-R\textsubscript{1} receptor is coupled to the particulate guanylate cyclase, while the ANP-R\textsubscript{2} receptor is non-coupled (18). Molecular cloning studies have identified two distinct ANP-R\textsubscript{1} receptors, such as the ANP-A and the ANP-B receptors (19, 20). ANP and BNP can only bind and stimulate guanylate cyclase of the ANP-A receptor, while CNP can only bind and stimulate that of the ANP-B receptor (21). Since the expression of CNP and the ANP-B receptor is limited primarily to the nervous system (21), it is thought that ANP, BNP, and the ANP-A receptor are more important for the regulation of salt and water balance as well as blood pressure by the endocrine system. The ANP-R\textsubscript{2} receptor, also called the ANP-C receptor, does not activate guanylate cyclase, but may function through an inhibitory guanosine-nucleotide-binding regulatory protein (Gi) to inhibit adenylate cyclase (16). It has been proposed that the major role of this receptor in vivo is to clear ANP and its related peptides (22).

We have screened cell lines which predominantly express a single ANP receptor subtype (9). Here the receptor binding profiles and agonistic activities of AP-811 for each of the ANP-A and the ANP-C receptor subtypes have been studied using such cell lines. In addition, the biological activities of AP-811 were compared with those of natural natriuretic peptides (hANP, pBNP, MetSO-hANP) (2-4) and ANP-C receptor-selective agonists (MetSO-hANP and C-ANF4-23) (9, 22).

AP-811 can bind to the ANP-C receptor expressed in Hela cells with an affinity similar to those of natural ligands and known ANP-C receptor-selective agonists. Since AP-811, which is derived from the Phe8-Ile15 region of ANP, can primarily bind to the ANP-C receptor with similar affinity to that of hANP, it is suggested that this receptor only recognizes the Phe8-Ile15 region of ANP. This observation is in agreement with the results reported by Bovy et al., who found that the GRIDRIG sequence from rat ANP is a binding site for the ANP-C receptor (7). AP-811 significantly inhibited adenylate cyclase activity coupled to the ANP-C receptor in rVSMC with a lower potency than those of hANP and C-ANF4-23, indicating that AP-811 is a partial agonist for the ANP-C receptor. These results indicate that the Phe8-Ile15 region of ANP contains residues essential for agonistic activity at the ANP-C receptor, although the non-peptide part of AP-811 may affect the agonistic activity. hCNP could not inhibit adenylate cyclase activity, although hCNP could bind to the ANP-C receptor in Hela cells with high affinity, and it was reported that this peptide also bound to rVSMC (23). Comparing the sequence of hCNP with that of C-ANF4-23, a full agonist of this receptor, the Leu9-Leu11 region probably does not complement the agonist site of the ANP-C receptor.

hANP showed high binding affinity for the ANP-A receptor expressed on CPAE cells. AP-811, pBNP, MetSO-hANP and C-ANF4-23 also bound to the ANP-A receptor, but with lower affinity. It is speculated that the binding site on ANP for the ANP-A receptor includes the Phe8-Ile15 region, but the site may be significantly larger. This conclusion is supported by the fact that AP-811 and C-ANF4-23 cannot activate the guanylate cyclase of the ANP-A receptor. A candidate for part of the remaining binding site is Arg27 in the C-terminal tail of ANP. von Geldern et al. have reported that the terminal arginine as a part of a C-terminal mimic is important for both binding and agonistic activities in the small ANP analog, A68828 (24). Interestingly, this arginine is located close to the Phe8-Ile15 region in the solution conformation of hANP (12).

According to the structure, AP-811 may not be hydrolyzed by aminopeptidases or carboxypeptidases, which can metabolize peptides in vivo. Recently it has been found that the major enzyme which hydrolyzes ANP in vivo is neutral metalloendopeptidase (NEP) (25). Considering the specificity of this enzyme, AP-811 may not be a substrate of NEP. These results suggest that AP-811 may have a longer duration than ANP when administered to animals. However, since AP-811 is an ANP-C receptor-selective agonist, this molecule would not have strong activities like those of ANP in vivo. Instead, it is expected that AP-811 may potentiate the biological activities of endogenous ANP, BNP and CNP in a similar manner to MetSO-hANP and C-ANF4-23 (9, 22), since AP-811 selectively blocks the ANP-C receptor, a clearance receptor of ANPs. According to our preliminary results, AP-811 may potentiate the hypotensive activity of co-administered hANP. Detailed studies of AP-811 in vivo will be described elsewhere.

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REFERENCES


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