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Research Article

The Interaction of Fatty Acid Amide Hydrolase (FAAH) Inhibitors with an Anandamide Carrier Protein Using ^{19}F-NMR

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Abstract. It has been reported that the endocannabinoid anandamide (AEA) binds to a class of fatty acid-binding proteins and serum albumin which can serve as carrier proteins and potentiate the cellular uptake of AEA and its intracellular translocation. Here, we employed ^{19}F nuclear magnetic resonance spectroscopy to study the interactions of serum albumin with two inhibitors of fatty acid amide hydrolase (FAAH), the enzyme involved in the deactivation of anandamide. We found that, for both inhibitors AM5206 and AM5207, the primary binding site on serum albumin is drug site 1 located at subdomain *IIA*. Neither inhibitor binds to drug site 2. While AM5207 binds exclusively to drug site 1, AM5206 also interacts with other fatty acid-binding sites on serum albumin. Additionally, AM5206 has an affinity for serum albumin approximately one order of magnitude higher than that of AM5207. The data suggest that interactions of FAAH inhibitors with albumin may provide added advantages for their ability to modulate endocannabinoid levels for a range of applications including analgesia, antiemesis, and neuroprotection.

KEYWORDS: anandamide carrier proteins; FAAH; ^{19}F-NMR; serum albumin.

INTRODUCTION

Fatty acid amide hydrolase (FAAH) is a membrane-associated enzyme (1–3) that catalyzes the hydrolysis of the endocannabinoid anandamide (*N*-arachidonoylethanolamine; AEA) and other bioactive amides (1–6). The blockade of FAAH can lead to chronic elevation of endocannabinoid levels at the synapse and produce sustained analgesic responses that are devoid of adverse effects typically associated with classical cannabinergic agonists (7–10). Therefore, FAAH has been a target of intense research efforts aimed at developing potent and selective inhibitors. Recently, it was reported that the cellular uptake of AEA can also be significantly potentiated by a class of anandamide carrier proteins (11–13) such as serum albumin, heat shock proteins (Hsp70), and fatty acid-binding proteins. These findings provide an opportunity for a potential new therapeutic modality to the treatment of pain through dual inhibition of FAAH and anandamide carrier proteins.

In an effort to explore the role of carrier proteins in the transport of endocannabinoids, we studied the interaction of two selective FAAH inhibitors, AM5206 and AM5207 (Fig. 1), with a representative anandamide carrier protein serum albumin. These two trifluoromethyl ketone analogs were selected because they represent a new generation of reversible FAAH inhibitors that can cross the blood–brain barrier and protect against the neurodegenerative changes and cytoskeletal damages (14–17). Serum albumin, the major protein constituent of blood plasma, has long been regarded as a reservoir and a carrier protein for various lipophilic ligands to their specific targets (18–26). The crystal structure of serum albumin reveals that it is a globular "heart-shaped" protein consisting of three homologous subdomains (*I*, *II*, and *III*) (22,27–29). There are two major structurally selective drug-binding sites (drug site 1 and drug site 2) located within subdomains *IIA* and *IIIA*, respectively (19,27,30–32).

The absence of fluorine in proteins provides much simplified ^{19}F nuclear magnetic resonance (^{19}F-NMR) spectra when studying the interactions between ^{19}F-labeled ligand and its target protein (33–38). Fluorine chemical shift anisotropy and exchange for screening has been commonly used as a rapid high-throughput screening NMR method to rank ligands for their binding affinity to a target protein (33,34). Here, our study was aimed at a more detailed characterization of the specific interactions between albumin and two of our lead FAAH inhibitors. We first employed

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ABBREVIATIONS: AEA, *N*-arachidonoylethanolamine or anandamide; BSA, bovine serum albumin; FAAH, fatty acid amide hydrolase.

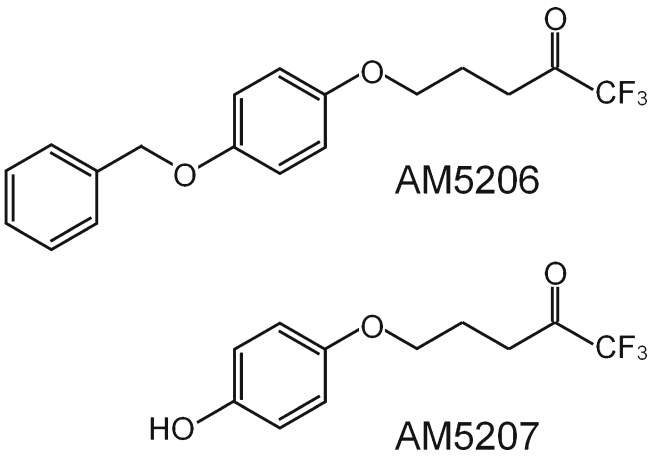

Fig. 1. Structure of the two trifluoromethyl ketone FAAH inhibitors, AM5206 and AM5207

fluorine NMR competition binding experiments to study the site-specific binding preference of these two FAAH inhibitors using site markers warfarin, L-tryptophan, and oleic acid (29,39–41). The binding affinity of each ligand was determined based on ^{19}F pulsed-field gradient (PFG) NMR diffusion measurements (42).

MATERIALS AND METHODS

Materials

Fatty acid free bovine serum albumin (BSA), warfarin sodium, L-tryptophan, and oleic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The two FAAH inhibitors AM5206 and AM5207 (Fig. 1) are trifluoromethyl ketone analogs synthesized in our laboratory, and the details of their synthesis will be published elsewhere. Deuterated dimethyl sulfoxide (DMSO) and deuterium oxide (D_2O) were purchased from Cambridge Isotope Laboratories, Inc. (Cambridge, MA, USA).

Sample Preparation

BSA was dissolved in D_2O, and the pH was 5.3 in concentrations ranging from 0.076 mM (0.5%) to 0.6 mM (4.0%). The two FAAH inhibitors AM5206 and AM5207 and the competing site markers warfarin, L-tryptophan, and oleic acid were separately prepared in concentrated stock solutions in DMSO-d_6 or D_2O. For the NMR diffusion experiments, each of the two FAAH inhibitors was added to BSA solutions and sonicated for 15 min in a water bath sonicator before experiments. The molar ratios of protein to each FAAH inhibitor are from 0.1 to 0.9. In the competitive binding experiments, each of the competing site markers was added from their respective stock solutions. The final concentrations of each competing site marker were 1, 3, 5, or 10 mM.

NMR Spectroscopy

All ^{19}F-NMR experiments were carried out at 376.5 MHz using a Bruker Avance II 400 MHz NMR spectrometer. All spectra were acquired using a 9.5-μs 90°-pulse and a 2-s repetition time without proton decoupling. The ^{19}F-NMR chemical shifts were referenced to an external standard (CF_3COOH, −76.55 ppm). Diffusion measurements were performed using the stimulated echo (STE)-PFG pulse sequence (42–45), with a maximum gradient of 0.324 T/m. These STE experiments were designed to achieve an accurate diffusion measurement by minimizing the T_2 relaxation effects (42).

RESULTS AND DISCUSSION

Site-Selective Binding of AM5206 and AM5207

In aqueous solution, the trifluoromethyl group on both AM5206 and AM5207 shows a single sharp ^{19}F-NMR resonance at −85.45 ppm (Figs. 2 and 3). Upon addition of BSA, the signal was significantly broadened with a downfield shift to −84.35 ppm for AM5206 and −84.83 ppm for AM5207. The significant line broadening suggests a much slower motion of both ligands after binding to the protein. The resonance at −85.45 ppm disappears completely, an indication that the exchange rate between the bound and unbound FAAH inhibitors in BSA solution was fast enough on the ^{19}F-NMR timescale.

To further compare the site-selective binding of AM5206 and AM5207, we have employed a series of competition binding experiments using three well-characterized site markers: warfarin, L-tryptophan, and oleic acid. Warfarin is known to have high binding affinity to drug site 1 at subdomain *IIA* (39). L-Tryptophan is a typical ligand for drug site 2 at subdomain *IIIA* through hydrogen bonding and hydrophobic and electrostatic interactions (40,41). The long chain fatty acid, oleic acid, primarily binds to domain *I* and domain *III* on albumin (29). Figure 2 displays the ^{19}F-NMR spectra of AM5207 in 0.6 mM BSA solution in the presence of warfarin, L-tryptophan, or oleic acid as a competing site marker. With increasing concentration of warfarin, the ^{19}F-NMR signal shifts upfield toward −85.45 ppm. This clearly demonstrates that AM5207 can be displaced by warfarin,

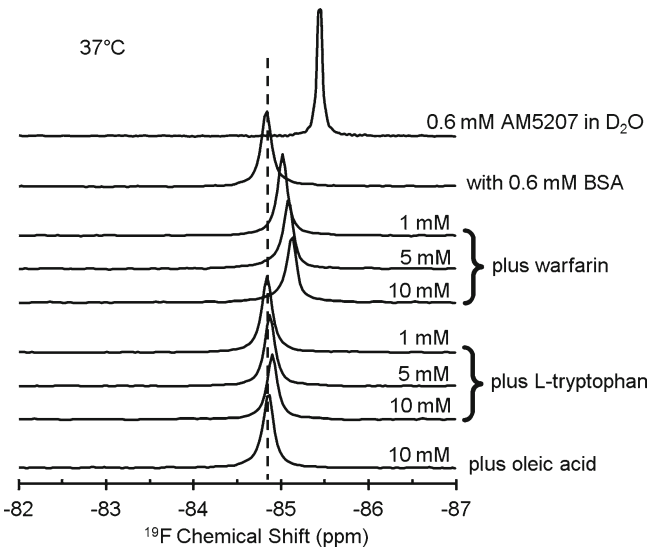

Fig. 2. ^{19}F-NMR spectra of 0.6 mM AM5207, with 0.6 mM BSA, and in the presence of three different competing site markers: warfarin, L-tryptophan, and oleic acid at 37°C

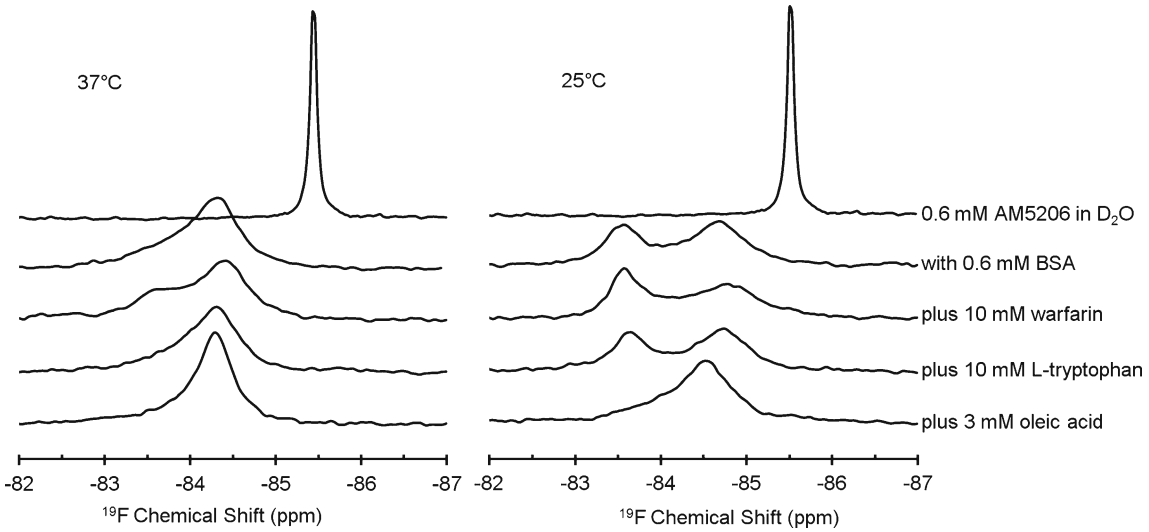

Fig. 3. ^{19}F-NMR spectra of 0.6 mM AM5206, with 0.6 mM BSA, and in the presence of three different competing site markers: warfarin, L-tryptophan, and oleic acid at 37°C (*left panel*) or 25°C (*right panel*)

indicating that AM5207 binds to drug site 1 at subdomain *IIA*. On the other hand, there are no discernible changes in the ^{19}F-NMR signal after adding L-tryptophan or oleic acid to the sample solution. We conclude that AM5207 primarily binds to drug site 1 at subdomain *IIA*, but not drug site 2 at subdomain *IIIA* or other domains on albumin.

Figure 3 shows the ^{19}F-NMR spectra of a series of competition binding experiments of AM5206 in BSA solutions at two different temperatures. All spectra on the left panel were recorded at 37°C. The addition of warfarin produces a shoulder at −83.55 ppm in the ^{19}F-NMR spectrum. However, no significant changes were observed with the addition of L-tryptophan. The ^{19}F-NMR signal becomes significantly narrower after the addition of oleic acid to the solution. This observation indicates a more complicated binding scenario that AM5206 may have redistributed between different binding sites with the addition of a competing site marker. Indeed, after decreasing the temperature to 25°C, we can clearly detect two well-resolved resonances (−84.75 and −83.55 ppm) due to AM5206 in BSA solutions, indicating at least two different binding sites for AM5206 on BSA. This is understandable because the exchange rate between the bound states is significantly decreased from 37°C to 25°C. When using warfarin as a competing ligand, the signal at −84.75 ppm decreases, while the signal at −83.55 ppm increases. This demonstrates that a portion of AM5206 binds to drug site 1 at subdomain *IIA* and thus can be displaced by warfarin. On the other hand, the addition of L-tryptophan does not produce any changes in the spectrum, indicating that AM5206 does not bind to drug site 2 at subdomain *IIIA*. Upon the addition of 3 mM oleic acid, the signal at −83.55 ppm almost completely disappears, while the signal at −84.75 ppm increases correspondingly. This illustrates that the signal at −83.55 ppm corresponds to AM5206 at some of the fatty acid-binding sites and thus can be displaced by oleic acid.

The binding characteristics of AM5206 were further studied by exploiting the two well-resolved resonances at 25°C with a series of titration experiments. Figure 4 shows the ^{19}F-NMR spectra with various BSA to AM5206 ratios. At a ratio of 1:0.5, the signal at −83.55 pm is much stronger than that at −84.75 ppm. With the addition of AM5206, the resonance at −84.75 ppm drastically increases. At a BSA/AM5206 ratio of 1:5, the signal at −84.75 ppm is ~10 times stronger than that of −83.55 ppm. Based on earlier competition binding experiments, we have assigned the −84.75 ppm peak to AM5206 binding at drug site 1 (possibly undergoing fast exchange with non-specific binding sites) and the −83.55 ppm peak to AM5206 binding at fatty acid-binding sites. The titration experiments show that,

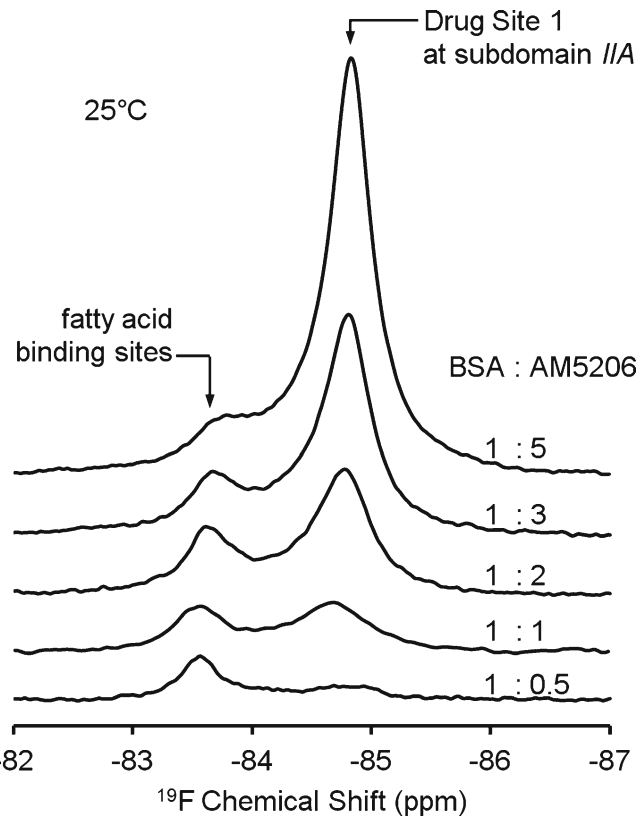

Fig. 4. ^{19}F-NMR spectra of AM5206 in 0.6 mM BSA solutions with various molar ratios of BSA and AM5206

while some of the fatty acid-binding sites can be easily accessible by AM5206 at low concentrations, drug site 1 at subdomain *IIA* is the primary binding site when there is an excess amount of ligand.

The Binding Affinity of AM5206 and AM5207 to Serum Albumin

NMR diffusion measurements can provide quantitative information on the ligand–protein interactions (42). Using ^{19}F STE-PFG NMR (42–45), the diffusion coefficients of FAAH inhibitors AM5206 and AM5207 were determined in the presence of BSA at different concentrations. This method involves acquiring a series of spectra with increasing gradient strengths. The signal intensity I can be described by (44):

$$\ln I/I_0 = -(\gamma g \delta)^2(\Delta - \delta/3)D \quad (1)$$

where I/I_0 is the spin-echo attenuation ratio of the signals in the presence and absence of gradient pulses, γ is the gyromagnetic ratio, g is the gradient strength, δ is the duration of the gradient pulses, Δ is the diffusion time, and D is the apparent self-diffusion coefficient. The top panels in Fig. 5 display the dependence of the apparent diffusion coefficient of AM5206 and AM5207 at different protein to ligand ratios ($[P_0]/[L_0]$). For both ligands, the apparent diffusion coefficient decreases with increasing protein to ligand ratio.

This set of diffusion coefficients allows us to determine the binding affinity (K_d) of each ligand to BSA. Under fast exchange conditions, the apparent diffusion coefficient (D) is the weighted average of two diffusion coefficients from ligand in its free and bound forms, which can be described as:

$$D = D_{bound} \times x_{bound} + D_{free} \times (1 - x_{bound}) \quad (2)$$

where x_{bound} is the fraction of the bound ligand and D_{bound} and D_{free} represent the diffusion coefficients of bound and free ligand, respectively. The bound fraction x_{bound} can be

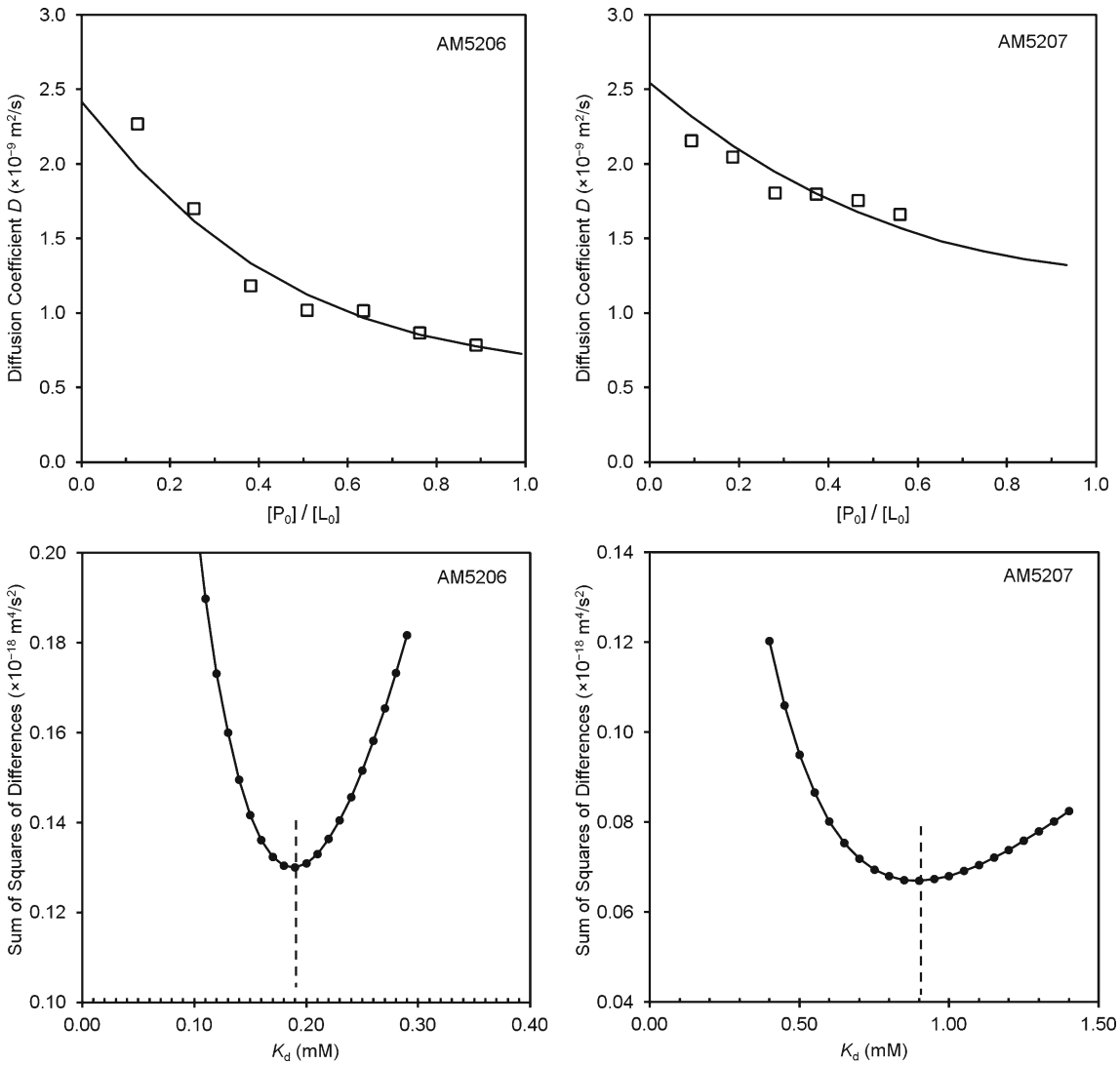

Fig. 5. Experimental and calculated diffusion coefficients as a function of protein to ligand ratio (*top panels*) and the determination of K_d values using least square curve fittings (*bottom panels*)

expressed in terms of the dissociation constant K_d, the number of binding sites n, and the total ligand and protein concentrations $[L_0]$ and $[P_0]$ (42):

$$x_{\text{bound}} = \frac{(n[P_0] + [L_0] + K_d) - \sqrt{(n[P_0] + [L_0] + K_d)^2 - 4n[L_0][P_0]}}{2[L_0]} \quad (3)$$

D_{bound} and D_{free} were obtained using similar ^{1}H or ^{19}F STE-PFG NMR measurements from pure protein and pure ligand preparations, respectively. For the number of binding sites n, it is reasonable to use n=2 for AM5206 in our calculations because it primarily binds to drug site 1, while a small fraction binds to the fatty acid-binding sites. On the other hand, we have used n=1 for AM5207 because it only binds to drug site 1. We can then obtain the dissociation constant (K_d) of AM5206 and AM5207 by fitting our experimental diffusion measurements using the least square method. The bottom panels in Fig. 5 are plots of the sum of squared differences between the experimentally determined diffusion coefficients (D) and the calculated D using a range of K_d values. For AM5206, the minimum squared error corresponds to the best fit for a two-site binding with a mean K_d value of 190 μM. Conversely, the best fit for AM5207 provides a K_d value of 900 μM with a single binding site on albumin.

CONCLUSIONS

The interactions of two trifluoromethyl ketone FAAH inhibitors with serum albumin were characterized by a series of competitive binding experiments and self-diffusion measurements using ^{19}F-NMR. We found that the primary binding site for both AM5206 and AM5207 is drug site 1 located at subdomain *IIA*. Neither of these two FAAH inhibitors binds to drug site 2 on albumin. While AM5207 binds exclusively to drug site 1, AM5206 also interacts with other fatty acid-binding sites on albumin. Interestingly, AM5206 tends to first bind to these fatty acid-binding sites especially at lower ligand concentrations, suggesting a much lower energy barrier for the ligand to access these sites. Furthermore, AM5206 has an affinity for serum albumin approximately one order of magnitude higher than that of AM5207. Arguably FAAH inhibitors such as AM5206 may compete with anandamide for binding with albumin and reduce the uptake of anandamide into cells. Such FAAH inhibitors can, thus, act by elevating the levels of available anandamide at the synapse through two converging mechanisms: the inactivation of FAAH and the inhibition of carrier protein-mediated transport of anandamide.

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