Binding and In Vitro Activities of Peptides with High Affinity for the Nociceptin/Orphanin FQ Receptor, ORL1

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ABSTRACT

Fifteen hexapeptides having high affinity for the opioid-like receptor ORL1 were identified from a combinatorial library containing more than 52 million different hexapeptides. The five compounds with the highest affinity were characterized further by use of a variety of in vitro models. Binding studies indicated that these five peptides have affinity for ORL1 in the nanomolar range, similar to the recently discovered endogenous ligand called nociceptin and orphanin FQ (N/OFQ). The activity of these compounds was investigated in three different assays: stimulation of [35S]GTPγS binding and inhibition of forskolin-stimulated cAMP accumulation in Chinese hamster ovary cells transfected with ORL1, and inhibition of electrically induced contractions in the mouse vas deferens. In each assay, the five hexapeptides acted as partial agonists. The EC50 values for stimulation of [35S]GTPγS binding and inhibition of cAMP accumulation were in the range of that for N/OFQ, but maximal effects ranged from 70 to 90% of N/OFQ in the cAMP assay, and 30 to 60% of N/OFQ in the GTPγS assay. The positive hexapeptides identified were found to have minimal structural similarity to N/OFQ. The peptides are positively charged, which could enable them to bind to the negatively charged second extracellular loop thought to be a likely binding site for N/OFQ.

The cloning of the opioid receptors led to the discovery by several groups of an additional receptor from this family (Bunzow et al., 1994; Lachowicz et al., 1995; Mollereau et al., 1994; Wang et al., 1994). Although this receptor, referred to here as ORL1 after Mollereau et al. (1994), has very high homology with the opioid receptors, the receptor transfected into mammalian cells did not bind opiates with the expected high affinity. This suggested the presence of an unknown endogenous neurotransmitter as its natural ligand. This endogenous ligand was subsequently identified by two groups independently (Meunier et al., 1995; Reinscheid et al., 1995) and was found to be a heptadecapeptide, with a sequence most closely resembling the opioid peptide dynorphin. The peptide was named orphanin FQ by Reinscheid et al. to designate the fact that it was an endogenous ligand for an orphan receptor and that its first and last amino acids were phenylalanine and glutamine. Meunier and colleagues named it nociceptin as an indication of its in vivo activity, because it was discovered that intracerebroventricular injection into mice led to a decrease in the hot-plate escape jumping latency (Meunier et al., 1995) and tail-flick latency (Reinscheid et al., 1995).

The in vitro activity of N/OFQ, as determined in cells transfected with ORL1, was as expected for an opioid-like receptor. N/OFQ, at low concentrations, inhibits forskolin-stimulated cAMP accumulation and stimulates [35S]GTPγS binding in brain membranes and transfected cells (Sim et al., 1996; Adapa and Toll, in press). Radioreceptor assays have been developed by use of both iodinated Tyr14-N/OFQ (Reinscheid et al., 1995) and the tritiated version of the Tyr14 analog (Adapa and Toll, in press), and the native peptide (Dooley and Houghten, 1996). Both peptides have been shown to bind with high affinity to transfected cells and brain membranes, and the binding properties seem to be very similar.

Unlike its close relatives the opioid receptors, very little is known of the pharmacology of ORL1. Meunier et al. (1995) demonstrated that the Tyr1 analog behaves very much like the native peptide, as does the Tyr14 analog (Reinscheid et al., 1995). The first structure-activity studies with truncated versions of N/OFQ have demonstrated that the amino-termi-
nal portion of the peptide is essential for high binding affinity and that the four C-terminal amino acids are not necessary for binding (Dooley and Houghten, 1996; Reinscheid et al., 1996). Although the sequence of N/OFQ has striking similarity to opioid peptides, and in particular dynorphin, the dynorphin gene products have only moderate affinity for ORL1, ranging from 100- to 1000-fold lower affinities than for the kappa opioid receptor (Adapa and Toll, in press). The development of new agonists and antagonists is of high priority if the in vivo functions of ORL1 and N/OFQ are to be better understood.

One method that has been very successful in the identification of novel peptidic and nonpeptidic structures that bind to opioid and other receptors has been the use of SCL (Houghten et al., 1991). SCLs are composed of mixtures of large numbers of compounds (e.g., 50 million, representing all possible combinations of the building blocks used) which are tested as mixtures. One of the major advantages of the SCLs is that the compounds are not support-bound and thus are tested as mixtures. One of the major advantages of the SCLs is that the compounds are not support-bound and thus can be used directly in solution for binding or functional assays (Houghten et al., 1992). Houghten and colleagues (1991) originally demonstrated the use of an SCL to identify peptides which corresponded to the antigenic determinant of an antibody. The use of an SCL to identify peptides closely related to the natural enkephalins was demonstrated in an opioid receptor assay (Houghten et al., 1992; Houghten and Dooley, 1993). Novel peptide antagonists to the mu receptor, the Acetalin (Dooley et al., 1993) and an all-D-amino acid agonist (Dooley et al., 1994) were also identified through the use of combinatorial libraries. Deconvolution, or identification of individual compounds from the complex mixtures in the libraries, can be achieved through an iterative process (described in the papers mentioned above) or by using a positional scanning library (Pinilla et al., 1992; Dooley and Houghten 1993). A PS-SCL enables the determination of the most active amino acids or building block at each position of a peptide or nonpeptide directly from the initial screening data. With the use of peptides as an example, PS-SCLs are composed of individual positional SCLs in which one or two positions are defined with a single amino acid, whereas the remaining positions are composed of mixtures of amino acids. The defined position(s) is “walked” through the entire sequence of the PS-SCL. It should be noted that each positional SCL, although addressing a single position of the sequence, represents the same collection of individual peptides. When used in concert, the data derived from each positional SCL yield information about the most important amino acids for every position. This library can be screened, and data obtained, in as little as a single assay. This information can then be used to synthesize highly active individual compounds. The work presented here describes the identification and properties of five hexapeptides having high affinity for ORL1, identified from a PS-SCL composed of more than 52 million hexapeptides.

Methods

Cell Culture

ORL1-containing CHO cells came from two sources. The rat receptor in CHO cells were obtained from Dr. Ping Law (University of Minnesota). The cDNA for the mouse receptor was obtained from Dr. Brigitte Kieffer (University of Strasbourg). The mouse ORL1 was subcloned into pcDNA3.1/His (Invitrogen, San Diego, CA) and this vector was transfected into CHO cells. pcDNA3.1/His produces a fusion protein containing a poly-His region and an antigenic epitope for commercial antibodies on the amino terminus of the protein. We have discovered no apparent effect of the amino-terminal tag on receptor binding or function, and the entire fusion protein has been used for the experiments described herein.

The cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes. For binding assays, the cells are scraped off the plate at confluence. For determination of inhibition of cAMP accumulation, cells were cultured onto 24-well plates and used at confluence.

Receptor Binding

Binding to brain membranes. Binding to brain membranes was conducted as described previously (Dooley and Houghten, 1996). Rat brains, (Harlan, Indianapolis, IN) minus cerebella, were homogenized in 40 ml of Tris buffer (50 mM Tris, 2 mM ethylenediaminetetraacetic acid, 100 mM phenylmethylsulfonyl fluoride, pH 7.4). Homogenates were spun for 10 min at 38,000 × g (Beckmann H2-JC, Fullerton, CA). Pellets were resuspended and incubated at 37°C for 30 min, and centrifuged for 10 min. Pellets were resuspended in 100 volumes of buffer, and bovine serum albumin (2 mg/ml) was added. Competition experiments were carried out in the presence of 3 nM [3H]N/OFQ (33 Ci/mmol) in a final volume of 0.65 ml. The reaction was terminated by filtration by a Tomtec 96 harvester (Orange, CT) through GF-B filters, previously soaked in 0.1% polyethyleneimine. Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ) and expressed in counts per minute. IC50 values were determined with six concentrations of each peptide analog, and calculated by use of Graphpad/Prism (ISI, San Diego, CA).

Binding to cell membranes. Binding to cell membranes was conducted in a 96-well format. Cells were removed from the plates by scraping with a rubber policeman, homogenized in Tris buffer with a Polytron homogenizer, then centrifuged once and washed by an additional centrifugation at 27,000 × g for 15 min. The pellet was resuspended in 50 mM Tris, pH 7.5, and the suspension incubated with [3H]N/OFQ (120 Ci/mmol) in a total volume of 0.2 ml for 15 min at 25°C. Samples were filtered and counted as described above.

Inhibition of forskolin-stimulated cAMP accumulation. The potency for the inhibition of forskolin-stimulated cAMP accumulation was conducted basically as described previously by Meunier et al. (1995) in intact CHO cells plated on 24-well plastic plates. To the cells was added [3H]adenine (1.0 µCi) in 0.4 ml of culture medium. The cells remained at 37°C for 2 h to allow the adenine to incorporate into the intracellular ATP pool. After 2 h, the cells were washed once with incubation buffer containing: 130 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.3 mM CaCl2, 1.2 mM MgSO4, 10 mM glucose, 1 mg/ml bovine serum albumin and 25 mM HEPES, pH 7.4, and replaced with buffer containing forskolin (10 µM) and isobutylmethylxanthine (50 µM). After 10 min, the medium was aspirated and replaced with 0.5 ml, 0.2 M HCl. Approximately 1000 cpm of [3H]cAMP was added to each well and used as an internal standard. The contents of the wells were then transferred to columns of 0.65 g dry alumina powder. The columns were eluted with 4 ml of 5 mM HCl, 0.5 ml of 0.1 M ammonium acetate, then two additional milliliters of ammonium acetate. The final eluate was collected into scintillation vials and counted for 14C and tritium. Amounts collected were corrected for recovery of [14C]cAMP.

[85S]GTPγS binding. [35S]GTPγS binding was conducted basically as described by Traynor and Nahorski (1995). Cells were scraped from tissue culture dishes into 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid, then centrifuged at 500 × g for 10 min. Cells were resuspended in this buffer and homogenized with a Poly-
tron Homogenizer. The homogenate was centrifuged at 27,000 g for 15 min, and the pellet resuspended in buffer A, containing: 20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, pH 7.4. The suspension was recentrifuged at 20,000 g and suspended once more in buffer A. The pellet was sometimes frozen at −70°C before use. For the binding assay, membranes (8–15 μg protein) were incubated with [³⁵S]GTPγS (50 pM), GDP (10 μM) and N/OFQ, in a total volume of 1.0 ml, for 60 min at 25°C. Samples were filtered over glass fiber filters and counted as described for the binding assays. Statistical analysis was conducted with the program Prism.

Mouse vas deferens assays were conducted as described previously (Berzetei-Gurske et al., 1996).

[³²H]N/OFQ (120 and 33 Ci/mmol respectively) were synthesized and generously donated by New England Nuclear/Dupont (Boston, MA), or synthesized as described previously (Dooley and Houghten, 1996). N/OFQ was from Phoenix Pharmaceuticals (Belmont, CA); Tyr₁⁴-N/OFQ was synthesized by Research Genetics (Huntsville, AL); and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

**Results**

A hexapeptide PS-SCL containing two defined positions was screened in the assay for N/OFQ. This PS-SCL can be represented by the formula (Ac-O₁O₂X₃X₄X₅X₆-NH₂, Ac-X₁X₂O₃O₄X₅X₆-NH₂, Ac-X₁X₂X₃X₄O₅O₆-NH₂), in which the positions labeled (O) are individually defined by the 20 natural L-amino acids (i.e., AA, AC, AD, . . . , YV, YW, YY). The positions labeled (X) consist of equimolar mixtures of 19 of the natural L-amino acids (omitting cysteine). This library contains 1200 mixtures (three sets of 400 mixtures each); each mixture contains 130,321 peptides (52,128,400 hexapeptides in total). Each set of 400 contains the same peptides, the difference being which positions are specifically defined. Upon screening the first sublibrary at 0.4 mg/ml (fig. 1A), which defined the first and second positions, only three mixtures inhibited 100% of [³²H]N/OFQ binding, Ac-AOXXXX-NH₂, Ac-COXXXX-NH₂ and Ac-DOXXXX-NH₂. Panel A represents the 400 mixtures in which the first and second positions are defined, each graph represents definition of the first position, each bar within a graph represents definition of the second position, thus the first bar of the first graph represents the mixture Ac-AAXXXX-NH₂, the second bar in the first graph represents the mixture Ac-ACXXXX-NH₂, etc. Panel B represents the 400 mixtures in which the third and fourth positions are defined. Panel C represents the 400 mixtures in which the fifth and sixth positions are defined.

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**Fig. 1.** Inhibition of [³²H]N/OFQ binding by mixtures from PS-SCL. Binding was conducted on rat brain membranes, as described under "Materials and Methods." Each peptide mixture contained 130,321 individual peptides and was used at a concentration of 0.4 mg/ml. Because we are interested in mixtures that inhibit virtually 100% of [³²H]N/OFQ binding, the y-axis represents 1% bound, so that 90% inhibition (10% bound) gives a value of 0.1, and greater than 90% inhibition gives a value of greater than 0.1. Panel A represents the 400 mixtures in which the first and second positions are defined, each graph represents definition of the first position, each bar within a graph represents definition of the second position, thus the first bar of the first graph represents the mixture Ac-AAXXXX-NH₂, the second bar in the first graph represents the mixture Ac-ACXXXX-NH₂, etc. Panel B represents the 400 mixtures in which the third and fourth positions are defined. Panel C represents the 400 mixtures in which the fifth and sixth positions are defined.
IC₅₀ values calculated for Ac-RFXXXX-NH₂ (IC₅₀ = 21 µM), Ac-RWXXXX-NH₂ (IC₅₀ = 21 µM) and Ac-RYXXXX-NH₂ (IC₅₀ = 8.5 µM) indicated that the third mixture was more active than the first two. All other mixtures had IC₅₀ values greater than 40 µM.

In the second sublibrary (fig. 1B), which defined the third and fourth positions, four mixtures inhibited more than 90% of [³H]N/OFQ binding (1/% Bound > 0.1): Ac-XXFRXX-NH₂, Ac-XXWRXX-NH₂, Ac-XXYKXX-NH₂ and Ac-XXYRXX-NH₂. The most active mixture was Ac-XXYRXX-NH₂, with an IC₅₀ of 24 µM. In the third sublibrary (fig. 1C), which defined the fifth and sixth positions, two mixtures inhibited more than 90% of [³H]N/OFQ binding: Ac-XXXXWK-NH₂ (92%) and Ac-XXXXWR-NH₂ (93%), with a third mixture Ac-XXXXIK-NH₂ inhibiting 75% of [³H]N/OFQ binding. Mixtures from the third sublibrary were less active; the most active mixture was Ac-XXXXWR-NH₂, which had an IC₅₀ of 91 µM, indicating that appropriate amino acids around the amino terminus are important for receptor binding. This is consistent with our previous findings with truncated N/OFQ peptides (Dooley and Houghten, 1996).

Table 1 shows Kᵢ values for each compound plus N/OFQ determined at the mouse ORL1 in CHO cells. In addition, table 1 lists the EC₅₀ value for stimulation of [³⁵S]GTPγS binding plus the percent maximal stimulation, as compared with N/OFQ, in these cells. Each novel peptide stimulated...
GTPγS binding at low concentrations, but none of the compounds stimulated to the extent of N/OFQ. Figure 2 shows this graphically and indicates the absolute amount of stimulated [35S]GTPγS binding. No binding of [3H]N/OFQ, nor stimulation of [35S]GTPγS binding was found in non-transfected CHO cells.

This partial agonist activity was also demonstrated in the other assays used. N/OFQ inhibited cAMP accumulation by an average of 84% in five experiments, with an IC50 of approximately 1.5 nM (table 2). Each of the peptides also inhibited cAMP accumulation, with approximately the same potency as N/OFQ. Once again none inhibited to the same maximal extent as N/OFQ, four of the peptides being significantly different in percent maximal inhibition.

The compounds were also tested in the MVD assay. We have previously shown that N/OFQ is able to block the electrically induced contractions of the MVD (Berzetei-Gurske et al., 1996). In the MVD assay, the compounds showed reasonably potent partial agonist activity. However, in this assay, the activity was difficult to determine because each of the peptides produced very strong tachyphylaxis. In other words, after each addition of the peptide, the vas deferens was unresponsive to an additional administration of the compound, or to the administration of N/OFQ (see fig. 3). We attempted to obtain IC50 values by extensive washing after each addition, but consistent data could not be generated. It was clear, however, that significant inhibition of the twitch response could be achieved at low concentrations, but high concentrations never produced the maximal inhibition level achieved by N/OFQ. One could tell that these actions were not through the opioid receptors because the agonist activity was not blocked by naloxone, and the tachyphylaxis induced by the peptides was selective for ORL1. After treatment with one of the ORL1 peptides, N/OFQ was no longer functional as an agonist, but the ability of the delta opioid agonist DPDPE to inhibit twitch was undiminished.

**Discussion**

The identification of novel ligands for receptors has traditionally been by standard medicinal or peptide chemistry, with new compounds based on the structure of known compounds. Particularly important for physiological studies would be the identification of high-affinity and stable analogs that could be used in vivo. This process may be difficult for larger peptides such as N/OFQ (FGGFTGARKSARKLANQ), because there are numerous potential sites not only for modification, but more importantly for degradation. N/OFQ also
TABLE 1
Binding affinities and stimulation of [35S]GTPγS binding by N/OFQ and peptides
Experiments were conducted on mouse ORL1 transfected into CHO cells. Data shown are the average ± SD of three experiments conducted in triplicate.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>[3H]N/OFQ Binding Ki (nM)</th>
<th>[35S]GTPγS Binding IC50 (nM)</th>
<th>% Maximal Stimulationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/OFQ</td>
<td>0.43 ± 0.05</td>
<td>4.0 ± 1.3</td>
<td>100</td>
</tr>
<tr>
<td>1 Ac-RYYRWR-NH2</td>
<td>0.60 ± 0.07</td>
<td>2.2 ± 0.2</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>2 Ac-RYYRK-NH2</td>
<td>0.71 ± 0.11</td>
<td>2.1 ± 1.3</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>3 Ac-RYYRIK-NH2</td>
<td>1.50 ± 0.11</td>
<td>5.1 ± 0.1</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>4 Ac-RYYKWR-NH2</td>
<td>1.47 ± 0.64</td>
<td>7.8 ± 4.1</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>5 Ac-RYYKWK-NH2</td>
<td>0.72 ± 0.21</td>
<td>6.7 ± 2.4</td>
<td>52 ± 6</td>
</tr>
</tbody>
</table>

a Ki values were calculated from the equation K = IC50/T + [L]/Kd. This equation appears valid because there seems to be a single binding site, with Hill coefficients close to 1.0. However, the reversibility of the synthetic peptides has not been determined, and the Ki values are based on the assumption that all components bind reversibly. The concentration of [3H]N/OFQ was 0.6 nM, and its Ki was determined to be 0.15 nM from direct saturation experiments.

b The percent maximal stimulation was relative to N/OFQ as 100% in each experiment. The percent maximal stimulation of each peptide was significantly different than N/OFQ, P > .05 by analysis of variance with post hoc Newman-Keuls.

Fig. 2. Stimulation of [35S]GTPγS binding by N/OFQ and five synthetic peptides. [35S]GTPγS binding was conducted on membranes (8.5 μg protein) from mouse receptor transfected into CHO cells, as described under “Materials and Methods.” Data shown are from a single experiment conducted in triplicate. The experiment was repeated twice with very similar results.

poses a particular problem because the truncation on the carboxy-terminal side produces inactive peptides when shorter than N/OFQ 1–14 (Dooley and Houghton, 1996). Furthermore, removal of the amino-terminal Phe (N/OFQ 2–17) produces an inactive compound (Matthes et al., 1996). It is the removal of Phe that is the initial and primary degradative step in processing N/OFQ (Yu et al., 1996).

Another, more novel method for the identification of new ORL1 ligands is the use of combinatorial libraries. In the present study, a peptide PS-SCL containing more than 150 million individual hexapeptides was used to identify compounds with affinities for ORL1 similar to that of N/OFQ. Because of the positional scanning nature of the libraries, this identification required the individual synthesis of only 20 peptides. Of these 20 peptides, five compounds with high affinity were studied further in three functional assays.

Each of the five peptides tested had high affinity for ORL1, and was a potent agonist in the inhibition of forskolin-stimulated cAMP accumulation and the stimulation of GTPγS binding in CHO cells transfected with ORL1. Although the concentrations in which the peptides produced their agonist activities in each of the systems were similar to that of N/OFQ, the maximal activity of each peptide was less than that of N/OFQ in each assay. For the rat receptor transfected into CHO cells, N/OFQ could induce a maximal 84% inhibition of forskolin-induced cAMP accumulation. The maximal inhibition of cAMP accumulation induced by the five peptides ranged from approximately 60 to 75%. This makes the peptides approximately 70 to 90% as efficacious as N/OFQ in this system. The reason for the partial agonist activity of these peptides is unknown. Although the activity of many peptide hormones can be mimicked by shorter peptides, or even non-peptides, it is possible that in this case, a peptide of greater than six amino acids would be required for full agonist activity. Although initial experiments were carried out on cells transfected with the rat receptor, receptor binding was difficult with these cells, as were the GTPγS binding studies (unpublished observation), probably because of a low receptor number. For this reason, we transfected CHO cells with the mouse receptor and obtained a clone for which the receptor number was quite high (1.2 pmol/mg) (Adapa and Toll, in press). Stimulation of GTPγS binding was conducted on these cells. In this system, each of the peptides also stimulated GTPγS binding at low concentrations, but once again, maximal activity was found to be significantly less than that found for N/OFQ. In this case the peptides ranged from 30% (peptide 3) to 60% (peptide 4) of the maximal [35S]GTPγS binding induced by N/OFQ.

The increased facility in demonstrating partial agonist activity in the GTPγS binding assay as opposed to the inhibition of cAMP accumulation is probably a function of the assay rather than the difference in species or potential differences in receptor number. First of all, the rat and mouse receptors are very similar, differing by one amino acid in the amino terminus and one in the carboxy terminus (Wick et al., 1994; Matthes et al., 1996), so it is unlikely that the primary sequence would affect the relative agonist activities. Second, the receptor number is higher in the CHO cells transfected with the mouse receptor. Finally, in the opioid system, the GTPγS assay is particularly adept at identifying partial agonist activity, which demonstrates partial agonist activity for morphine at the mu receptor (Traynor and Nahorski, 1995), although morphine is a prototypical full agonist in most in vivo and in vitro paradigms.

Each high-affinity peptide is very highly charged, containing three basic residues (Arg or Lys). In fact, in each sublibrary tested (Ac-OXIXXX-NH2, Ac-XXOXX-NH2, and Ac-
XXXOO-NH<sub>2</sub>), Arg was found to be a preferred amino acid in one of the defined positions. In this respect they are similar to N/OFQ, which has 4 positively charged amino acids among its 17 amino acids. These highly charged peptides would be expected to bind tightly to a potential N/OFQ binding site on the acidic amino acid-rich second extracellular loop of ORL1 (Meunier <i>et al.</i>, 1995). It is also noteworthy that these sequences bear modest resemblance to the Acetalins, Ac-RFMWMK-NH<sub>2</sub>, however the Acetalins were found to bind poorly to ORL1 and the five peptides described here bound poorly (>4000 nM) in mu, delta and kappa opioid receptor assays.

In conclusion, these studies demonstrate the utility of combinatorial chemistry for the identification of novel ligands for a G protein-coupled receptor. The five high-affinity hexapeptides represent the first small peptides with high affinity for ORL1 and identify not only potentially useful ligands for the investigation into the function of ORL1, but also an improved starting place for the discovery of new ligands. The investigation of the <i>in vivo</i> activity of these hexapeptides is currently underway.

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References

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